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Germination-Enhancing Techniques for *Pinus monticola* Seeds

Final Research Report to the USDA Forest Service, Intermountain Research Station

R. Kasten Dumroese and David L. Wenny

Forest Research Nursery, University of Idaho, Moscow

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ABSTRACT

A papery-layer in seeds of *Pinus monticola* Dougl. ex D. Don delays germination and reduces efficient use of seeds in nurseries. Six experiments, including exposing mega-gametophytes by removing portions of seed coats and papery-layers (clipping), warm-moist treatments before stratification, accelerated aging after stratification, soaking seeds in ethylene inhibitors or precursors before stratification, varying soaking durations, and excising embryos of nonstratified seeds, were used to improve the understanding of environmental effects on seed germination. Germination of embryos excised from nonstratified seeds was 99% seven days after excision, compared to 50% germination of embryos retained in megagametophytes and zero germination of intact seeds. Placing exposed megagametophytes of nonstratified seeds toward air resulted in 27% more seed germination than when seeds had exposed megagametophytes placed toward a moist substrate; after 120 days stratification seed germination was similar, regardless of megagametophyte exposure. After a stratification duration $\leq$ 45 days, three times more clipped seeds germinated than control seeds. After 45 days stratification, twice as many warm-moist treated seeds germinated as control seeds. After 90 days stratification, $\approx$ 91% of the seeds that were clipped, warm-moist treated, or given both treatments germinated. Post-stratification exposure to warm temperatures and high humidity using accelerated aging techniques was ineffective at improving total germination. Seed moisture content and subsequent total germination increased as soaking duration increased from 0 to 72 h. Seed moisture content also increased as soaking temperature increased from 13 to 20°C. Treating seeds with ethylene precursors and inhibitors failed to affect germination.

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CHAPTER I

SEED DORMANCY IN *PINUS* SPP.

A Review of the Literature

Spreading seed germination over time is ecologically important as it increases the survival of a species. Retention of portions of a seed population allows some progeny to survive environmental conditions adverse to emerging seedlings (Mayer and Poljakoff-Mayber 1989), essentially allowing seeds to disperse through time (Angevine and Chabot 1979; Chambers and MacMahon 1994). Seed retention from year to year is a common adaptation in many species (Koller 1972) and under genetic control (Naylor 1983).

DORMANCY CLASSIFICATION

Crocker (1916) introduced the concept of primary and secondary dormancy. Primary dormancy is defined as seeds failing to germinate immediately after being shed, though conditions for germination are favorable. Seeds able to germinate immediately after being shed, but without favorable conditions, enter secondary dormancy and will not germinate if favorable conditions return. An after-ripening process is needed before seeds with either primary or secondary dormancy germinate.

Hobson (1981) notes the definitions of dormancy may be as numerous as the investigators of this subject. Vleeshouwers et al. (1995) state that seed dormancy is any phenomenon within seeds that prevents germination, and clearly distinct from processes required to evoke germination. Both primary and secondary seed dormancy factors may be either exogenous or endogenous (Nikolaeva 1977). Endogenous dormancy includes morphological and physiological factors. Morphological dormancy is usually due to embryo immaturity, and a warm after-ripening period is needed to complete maturation. Physiological dormancy factors, common in forest tree species, especially *Pinus* spp. (Krugman and Jenkinson 1974), are removed by meeting metabolic requirements through a cold-moist, after-ripening treatment. Exogenous dormancy includes mechanical, physical, and chemical inhibitors. Physical constraints to germination are due to seed coat impermeability to water or oxygen, whereas mechanical dormancy is caused by a physical restraint of testa to enlarging or emerging embryonic tissue. The presence of chemical inhibitors constitutes chemical dormancy (Leadem 1987). Compounding all exogenous and endogenous factors are underlying genetic influences that can affect germination on a geographic (Fowler and Dwight 1964; McLemore and Barnett 1966; Heit 1973; Moore and Kid 1982; Richter and Switzer 1982; Weber and Sorensen 1992) and family basis (Andrews 1980; Carpita et al. 1983; Hoff 1987; El-Kassaby et al. 1993), and from various positions in the crown of a tree (Webb and Wareing 1972; Bramlett et al. 1983). Heydecker (1977) summarized dormancy complexity by writing, "This survival adaptation is a complex interaction between seed and environment, often involving more than one survival mechanism, sometimes intricately connected, sometimes parallel, and even with a certain mechanism simply not detrimental but without being of any clear positive advantage."

Nikolaeva's (1977) dormancy classification has utility. However, multiple factors may be responsible for dormancy, while relationships among these factors often make specific classifications difficult (Mayer and Poljakoff-Mayber 1989). Even broad classifications may significantly overlap to make them cumbersome and confusing. For example, endogenous and exogenous are perhaps the broadest categories of primary dormancy. However, if an embryo needs oxygen to complete an after-ripening phase (endogenous dormancy), while oxygen is restricted by testa (exogenous dormancy), what is the best seed dormancy classification?

Lang et al. (1987) proposed a classification system with nomenclature built systematically on the base term "dormancy," with dormancy defined as "a temporary suspension of visible growth of any

plant structure containing a meristem." The next level of nomenclature adds a descriptive, specific, physiological prefix to the word dormancy, depending on whether dormancy was induced by: 1) an unsuitable environmental factor (*eco*dormancy), 2) a physical or biochemical factor external to the embryo (*para*dormancy), or 3) physiological factors within the embryo (*endo*dormancy). The next level of nomenclature can be further refined by adding physiological descriptions (i.e. cryogenic when chilling, spectral when light, or atmospheric when either oxygen or carbon dioxide are required to dissipate dormancy). Using the previous example with endogenous and exogenous dormancy, a seed with an embryo needing oxygen but oxygen is restricted by testa would have *testa atmospheric paradormancy* using the system of Lang et al. (1987). For this review, I will focus on primary dormancy.

PRIMARY DORMANCY

Most *Pinus* spp. seeds have primary dormancy (Crocker 1916), although sometimes seeds from *P. banksiana* Lamb., *P. contorta* var. *latifolia* Engelm., *P. nigra* Arnold, *P. sylvestris* L., and *P. taeda* L. and may germinate well without any treatments. Since seeds from most *Pinus* spp. can be stimulated to germinate (or germinate faster) after cold-moist, after-ripening treatments (Krugman and Jenkinson 1974), their embryos are mature when shed. Mature embryos have been found in *P. contorta* var. *latifolia*, *P. lambertiana* Dougl., and *P. monticola* Dougl. ex D. Don seeds (Baron 1966; Pitel and Wang 1980; Leadem 1986). However, some seedlots of *P. albicaulis* Engelm. supposedly benefit from a warm after-ripening period, purportedly allowing the embryo to mature (Pitel and Wang 1980; Leadem 1986). Besides being morphologically mature, some *Pinus* spp. embryos lack dormancy since excised, nonchilled embryos of *P. lambertiana* (Haddock 1954; Sacher 1956; Baron 1966) and *P. taeda* grow (Carpita et al. 1983; Gifford 1993; Schneider and Gifford 1994). *Pinus taeda* embryos, despite the presence of megagametophyte or seed coat, germinate after exposure to chilling (Carpita et al. 1983). Primary dormancy associated with physiological factors accounts for 50% of *P. monticola* seed dormancy and is associated with the megagametophyte-embryo (Hoff 1986, 1987). For *P. monticola*, however, physiological dormancy can be overcome with a 42- to 90-day stratification[1] (Krugman and Jenkinson 1974; Hoff 1987). Stratification duration was the major factor influencing *P. strobus* L. speed of germination and total germination; stratification temperature is less important when maintained between 2-10°C (Graber 1965).

SEED COAT DERIVATION

Pinus spp. seed coats have distinct layers of integumental origin (Coulter and Chamberlain 1917; Quisumbing 1925; Chamberlain 1935). The proembryonic integument begins to differentiate into distinct layers shortly after fertilization. At maturity, the outer layer, sarcotesta (Figs. 1.1-1.2), comprises thin-walled, sometimes elongated cells in which ergastic materials, including tannins, accumulate. The inner stony layer, sclerotesta, is characterized by pitted, thick-walled cells. Usually, stony layer cells are most abundant in the micropylar region, abundant in the chalazal region and least

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[1] Evelyn (1664) first described alternating layers of seeds and moist soil in a barrel and exposing these strata to winter temperatures. Thus, *stratification* became the process by which seeds were artificially exposed to cold-moist conditions between layers of soil or peat to encourage subsequent germination in spring. Although researchers like Stone (1957) determined stratification could be duplicated without strata of soil or peat, the term *stratification* has become the definition for any cold-moist conditioning treatment that evokes germination (Krugman et al. 1974). Colloquially, stratification has also been used to describe any warm or cold moist-conditioning of seeds (Krugman et al. 1974), although the addition of warm conditions to the definition is confusing and unjustified. I use *stratification* to describe cold-moist treatments that evoke germination.

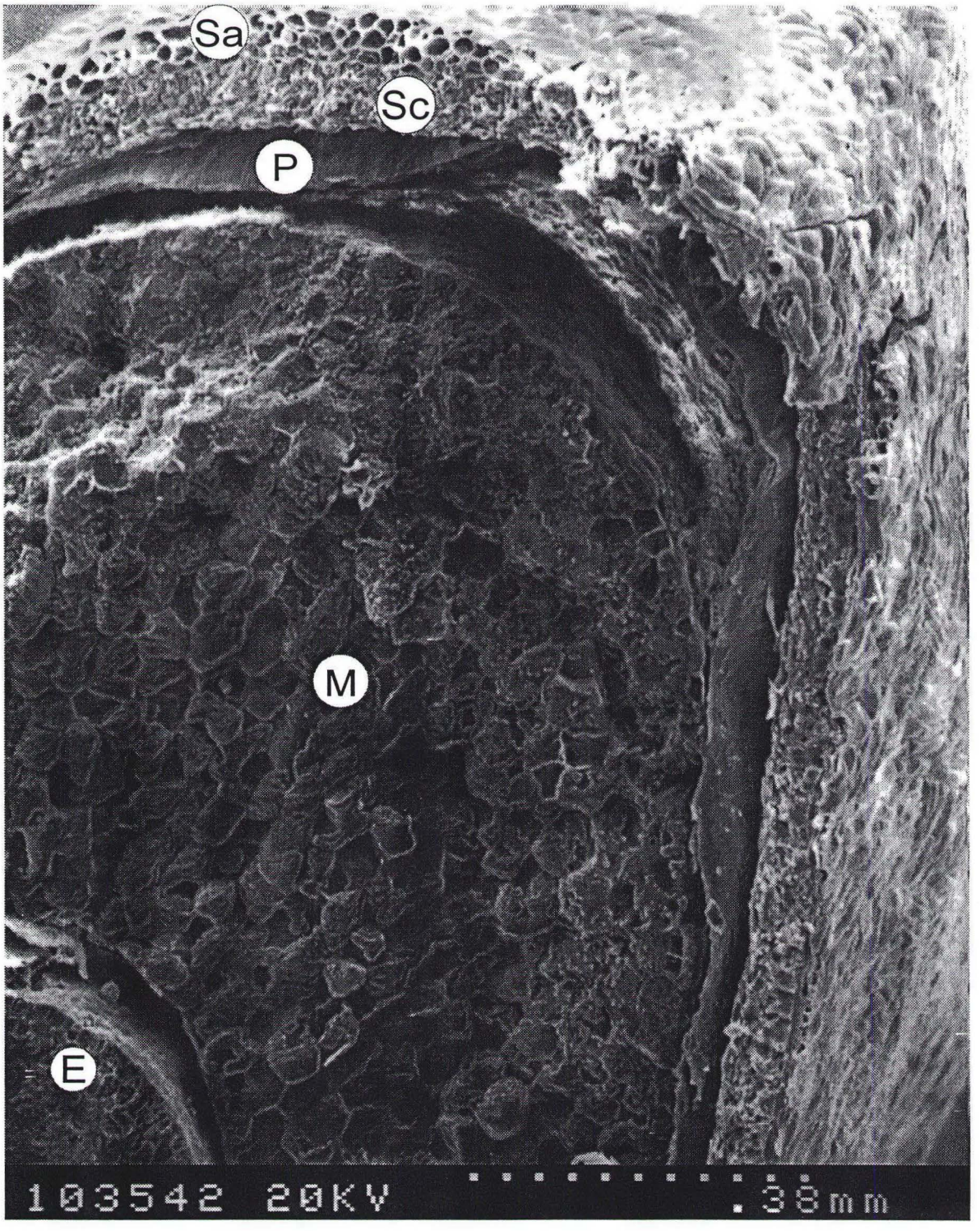

Figure 1.1. Scanning electron micrograph of a cross section of a *P. monticola* seed showing sarcotesta (Sa), sclerotesta (Sc), papery-layer (P), megagametophyte (M), and embryo (E).

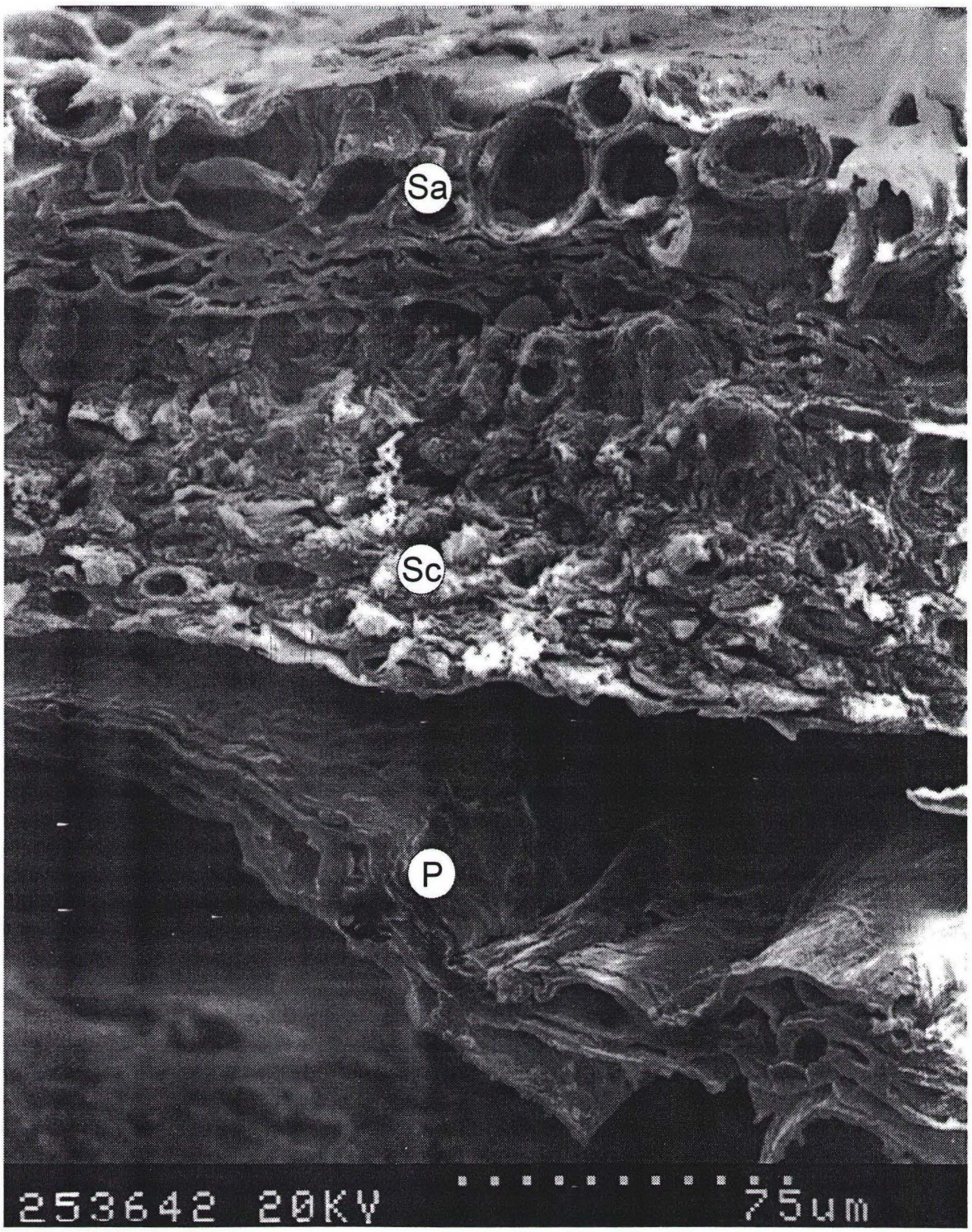

Figure 1.2. Scanning electron micrograph of a cross section of megagametophyte-covering layers: sarcotesta (Sa), sclerotesta (Sc), and papery-layer (P).

abundant along sides of seeds. The inner fleshy layer, endotesta, comprises elongated and flattened cells (Konar and Ramchandani 1958; Lill 1976; Owens and Molder 1977; Owens et al. 1982). In mature P. monticola seeds, sarcotesta comprises thin-walled, elongated cells containing ergastic material. Sclerotesta is characterized by small, thick-walled cells with dense cytoplasm and abundant starch. Endotesta is made of thin-walled, elongated, vacuolated cells (Owens and Molder 1977).

Classification of megagametophyte-covering layers is confusing (Tillman-Sutela and Kauppi 1995). Quisumbing (1925) attributed the "parchment-like" layer as the two inner layers of the three-layer integument. Owens and Molder (1977) identify it as endotestae. In *P. sylvestris* seeds, tissues occupying the cavity between the seed coat and megagameto-phyte are adnexal remnants, though it appears on macroscopic examination as a single, non-living papery-layer (Tillman-Sutela and Kauppi 1995). These adnexa are mostly nucellar, but the innermost layers represent megaspore membranes (exine and intine). Also included in this cavity are excretions of the tapetum. The papery-layer is attached to the chalazal end of seeds. Layers adjacent to the seed coat (nucellar) are more reticulate at openings in the seed coat, partially adnated endotesta, and extended through openings in sclerotesta and at the micropyle (Tillman-Sutela and Kauppi 1995). Although adnexa between seed coat and megagametophyte are not seed coat, the following discussion on seed coat-imposed primary dormancy will consider the papery-layer as part of the seed coat, since most literature is vague about the origin of megagametophyte-covering structures, and because the seed coat proper cannot be removed without disrupting the papery-layer (Baron 1966; Hoff 1987; Dong et al. 1993; Tillman-Sutela and Kauppi 1995).

SEED COAT-IMPOSED DORMANCY

The seed coat, along with the papery-like layer between the seed coat and mega-gametophyte, is considered a primary dormancy cause in *Pinus* spp. seeds (Stone 1957; Fowler 1959; Asakawa 1964; Baron 1966, 1978; Barnett 1972, 1976; Malone 1983, 1985; Hoff 1986, 1987; Pitel and Wang 1990; Dong et al. 1993). A few authors report improved seed germination by disrupting or removing the seed coat, without specifying whether or not the papery-layer was disturbed (Kozlowski and Gentile 1959; Andrews 1980; Murphy and Noland 1981a; Pitel and Wang 1985). Seed coat-imposed dormancy appears to limit water or gas exchange. Papery-layers of *P. lambertiana* (Baron 1978) and *P. sylvestris* (Tillman-Sutela and Kauppi 1995) were physical barriers to seed hydration. In *P. sylvestris*, papery-layers were lipophilic and much more effective than seed coats in retarding water passage to the megagametophyte; seed hydration rate differed between seed sources (Tillman-Sutela and Kauppi 1995). Thick seed coats of *P. albicaulis* were believed to restrict water entry (Taylor and Wareing 1979), gas exchange, or both (Pitel and Wang 1990). Asakawa (1956) assumed that thick seed coats of *P. koraiensis* Sieb. & Zucc. impeded seed hydration, but recent work indicates otherwise (Qi et al. 1993). Similarly, the seed coat of another thick-coated Asian pine (*P. bungeana* Zucc.) was permeable to water (Dong et al. 1993). In addition to impeding water entry, seed coats of *P. strobus* restricted gas movement into seeds and a lack of oxygen appeared to be limiting germination (Kozlowski and Gentile 1959). Water was rapidly lost from *P. strobus* seeds during drying, suggesting covering structures were very permeable to water (Downie and Bergstem 1991b). *Pinus jeffreyi* Grev. & Balf. seed coats restricted gas exchange but not water (Stone 1957). Barnett (1993) found *P. palustris* Mill. seeds soaked as little as four hours contained too much water and germination was reduced. Barnett (1972, 1976) reported mechanical dormancy in *P. taeda* because seed coats allowed initial water entry but restricted complete hydration of embryos and megagametophytes, a necessary condition for germination.

Seed coats accounted for about 9% of *P. monticola* dormancy while papery-layers were responsible for about 34% (Hoff 1987). Seed coat-imposed dormancy is removed by 21 days stratification

although the papery-layer is the primary dormancy source restricting germination after 90 and 105 days stratification (Hoff 1987).

Leadem (1986) concluded that *P. monticola* seed coats were mechanical barriers to germination because clipping seed coats (partial removal of the seed coat and papery-layer to expose megagametophyte tissue) at the micropylar end resulted in prompt germination. Pitel and Wang (1985) also thought clipping micropylar ends of *P. monticola* seed coats reduced mechanical resistance for expanding primary roots, but these researchers failed to rule out the possibility of improved gas exchange with the embryo. However, more recent work with seeds of *P. sylvestris* indicates that tissues at the micropyle, including the sclerotesta and nucellar cap, are the most impervious to water entry (Tillman-Sutela and Kauppi 1995). In contrast, when seed coats of nonstratified seeds were slit longitudinally exposing megagametophytes toward atmosphere or a moist substrate, more megagametophytes exposed to air germinated, indicating seed coats restricted gas exchange (Pitel and Wang 1985). Hoff (1987) argues, and others agree (Bewley and Black 1985), that because germination can be stimulated by a minor disruption of seed coats distant from the usual point of primary root emergence, seed coats act more as physical barriers to water or gas exchange than mechanical barriers to expanding embryonic tissues.

Seed coats might limit gas exchange in two ways: impeding oxygen entry or carbon dioxide escape (Bewley and Black 1985). In hydrated seeds, oxygen diffusion rate is already very low because gas must diffuse in solution (Werker 1981). If oxygen entry is further impeded, dormancy may be overcome by minor disruption of the integrity of the impeding layer, or by placing seeds into an oxygen-enriched atmosphere (Bewley and Black 1985). Seed coat disruption, including disruption of papery-layers, increased *P. monticola* seed germination (Pitel and Wang 1985; Leadem 1986; Hoff 1987). Decorticated *P. monticola* seeds also had increased respiration (Leadem 1993). Kozlowski and Gentile (1959) observed *P. strobus* seeds placed in an oxygen-enriched chamber had significantly increased respiration and germination. *Pinus clausa* var. *clausa* D.B. Ward seeds soaked in aerated water before stratification germinated at a higher percentage than control seeds (Outcalt 1991). Bewley and Black (1985) speculate this type of treatment gives embryos access to oxygen so germination can continue. However, to fully assess the plausibility of this view, we must know seed coat-permeability to oxygen, the oxygen threshold level needed by the embryo for germination, and whether seed coats do indeed restrict available oxygen below that necessary for germination. Currently, this information is lacking for *Pinus* spp. and other gymnosperms, and most forest trees. However, Bewley and Black (1985) conclude after reviewing literature for other higher plants that seed coats fail to impose dormancy by restricting oxygen for embryo respiration.

ROLES OF PLANT GROWTH REGULATORS & BIOCHEMICAL REACTIONS IN DORMANCY

Abscisic Acid, Gibberellins & Respiration

If embryos receive sufficient oxygen for respiration, what continues to inhibit germination? Perhaps plant growth regulators are acting on, or in concert with, specific protein synthesis and other biochemical reactions within the megagametophyte and/or embryo.

Seeds of boreal *Pinus* spp. can be dried to low moisture contents (3-6%) and stored below freezing temperatures to maintain viability for extended durations (Roberts 1973; Krugman and Jenkinson 1974). Biochemical reactions begin almost immediately when water enters dried seeds. Respiration in *P. monticola* seeds increased within minutes of water entry (Leadem 1993). Abscisic acid (ABA) or ABA-like compounds were found in *P. koraiensis* (Wang et al. 1982), *P. lambertiana* (Murphy and Noland 1981a, 1981b), and *P. pinea* L. (Martínez-Honduvilla and Santos-Ruiz 1978; Corvillon and Martínez-Honduvilla 1980). Although ABA is undoubtedly important in preventing vivipary (Quatrano 1987), its role as germination inhibitor in dispersed *Pinus* seeds is unlikely. Generally, *Pinus* seed

coats contain appreciable ABA but most is leached during seed hydration and continues to dissipate during cold-moist treatment (Martínez-Honduvilla and Santos-Ruiz 1978; Murphy and Noland 1981a).

Once seeds are hydrated and stratification begins, several processes begin. During stratification, *P. monticola* seed respiration remained constant (Leadem 1993), but increased in *P. lambertiana* seeds (Murphy and Hammer 1988). In *P. lambertiana*, ABA levels continue to decline in seed coats, megagametophytes, and embryos (Corvillon and Martínez-Honduvilla 1980; Murphy and Noland 1981a, 1981b). Although ABA levels in *P. lambertiana* embryos remained constant and higher than that in seed coats during the first 30 days of stratification, germination increased from 5% to 20% (Murphy and Noland 1981b). Further, exogenous ABA applications after 90 days stratification failed to inhibit *P. lambertiana* germination (Murphy and Noland 1981b). Either white- or red-light cause decreases in ABA concentrations before *P. sylvestris* seed germination (Tillberg 1992; Tillberg and Bjorkman 1993). Germination of *P. strobus*, *P. taeda*, and *P. virginiana* Mill. seeds was increased by red light, and effectiveness of red light increased with a longer stratification duration at 5°C (Toole et al. 1961, 1962). However, only nonstratified *P. contorta* var. *latifolia* and *P. ponderosa* Dougl. ex Laws. seeds have increased total germination after exposure to light (Li and Burton 1994).

As seed levels of ABA decline, amounts of gibberellins in *P. taeda* seeds increase (Paul et al. 1973). Although gibberellic acid (GA) is an endogenous component of many seeds, Leadem (1987) concludes only that exogenous GA can release conifer seeds from dormancy. The significance of the role GA plays in dormancy termination is unclear. Exogenous GA alone successfully improved total germination of *P. albicaulis* (Pitel and Wang 1990), *P. bungeana* (Dong et al. 1987), *P. strobus* (Jenkins 1961), *P. sylvestris* (Grover 1962; Sandberg 1988), and *P. taeda* (Biswas et al. 1972; Geyer 1974), and when combined with kinetin and stratification, improved *P. monticola* seed germination (Pitel and Wang 1985). However, exogenous GA failed to improve total germination and speed of germination of *P. edulis* Engelm. seeds (Gottfried and Heidmann 1992).

Sucrose & Protein Synthesis

During stratification, sucrose content of *P. taeda* megagametophytes decreases and embryos have increased capacity to metabolize sucrose (Carpita et al. 1983). Sucrose synthase activity and starch synthesis in *P. lambertiana* embryos and lipid metabolism in megagametophytes increase during stratification (Murphy and Hammer 1988, 1993).

For most plants, protein synthesis is obligatory for germination (Brooker et al. 1977). Although soluble protein content of embryos remains unchanged in *P. lambertiana* (Murphy et al. 1980) and *P. taeda* (Gifford 1993), embryos and megagametophytes of both species synthesize proteins at an increasing rate throughout their exposure to cold-moist conditions (Barnett et al. 1974; Noland and Murphy 1986; Gifford 1993; Schnieder and Gifford 1994). Protein band patterns of embryos and megagametophytes remained similar within stratified *P. lambertiana* seeds (Noland and Murphy 1986) as did patterns within *P. taeda* embryos (Gifford 1993). However, during stratification, synthesis of new proteins, evident by a shift in band patterns, was found for *P. taeda* megagametophytes; germination increased concurrently with this shift (Gifford 1993).

Ethylene & Adenosine Triphosphate

Heating seeds often increases germination and similar results can be accomplished using ethylene. Although Leadem's (1987) review of hormonal control of tree seed germination fails to discuss ethylene action, several investigators increased conifer seed germination with exogenous ethylene applications (Hamilton 1972; Borno and Taylor 1975; Shibakusa 1980). Furthermore, the effective ethylene concentration and treatment duration varied among species (Hamilton 1972), and seeds

stimulated by ethylene may have been a component of the population not released from dormancy by stratification alone (Borno and Taylor 1975).

Ethylene will induce dormant *Arachis hypogaea* L. seeds to germinate (Ketring and Morgan 1970) and similar results can be achieved by subjecting seeds to heat treatments. In addition, *Trifolium* spp. seeds were more responsive to ethylene at 20°C than 4°C (Esashi and Leopold 1969). Dormant *P. monticola* seeds react positively to some warm treatments. Anderson and Wilson (1966) improved *P. monticola* seed germination by exposing hydrated seeds to warm-moist conditions for 30 days before stratification. Similar results were found for *P. peuce* Grisebach seeds (Mason et al. 1995). In *P. strobus* seeds, a warm-moist treatment following stratification improved speed of germination, and this treatment alone increased total germination of nonstratified seeds (Downie and Bergstem 1991a). Soaking *P. taeda* seeds in 24°C water (Boyer et al. 1988) and *P. koraiensis* seeds in 70°C water (Qi et al. 1993) increased total germination. Subjecting *Pinus* spp. seeds to radio frequency dielectric heating, infrared radiation, or gas plasma radiation heated seeds to some degree and for *P. lambertiana*, *P. sabiniana* Dougl., and *P. strobus* increased germination over non-treated controls but less than stratification (Nelson et al. 1980). Dormant, hydrated *P. lambertiana* seeds exposed to warm temperatures for a few days had high rates of synthesis of proteins (Noland and Murphy 1986), adenosine triphosphate (ATP), and isocitrate lyase in megagametophytes and embryos, but the rates declined precipitously as the warm treatment continued (Noland and Murphy 1984). One possible, positive effect of osmotic priming on *P. banksiana* seed germination is the correlated increase in ATP availability (Bourgeois and Malek 1991).

Ethylene increases L-phenylalanine ammonia-lyase (PAL) activity (Prasad and Cline 1987). Stratification results in a four-fold increase in PAL activity in *P. lambertiana* embryos, apparently induced by cold temperature (Murphy et al. 1980). PAL activity indicates phenol metabolism, and indeed, levels of phenolic acids increase appreciably in stratifying *P. lambertiana* seeds (Murphy and Noland 1981a, 1981b). Phenolic compounds were proposed as germination inhibitors (Van Sumere et al. 1972), but Murphy and Noland (1981a, 1981b) failed to find evidence to support this proposal.

Indoleacetic acid (IAA) levels in *P. sylvestris* seeds increase sharply during hydration (Tillberg 1977). IAA increases ethylene production (Lieberman 1979), and ethylene production in seeds increases during hydration (Mayer and Poljakoff-Mayber 1989). Ketring and Morgan (1969) concluded that natural dormancy release in *A. hypogaea* was dependent on a seed's ability to increase ethylene production capacity and raise internal ethylene concentrations above a critical activation level. Ethylene may be necessary to stimulate embryo expansion to split seed coats (Abeles 1986; Schonbeck and Egley 1988) and may (Schonbeck and Egley 1988) or may not (Abeles 1986) assist with primary root penetration through endosperm. High oxygen tensions are necessary for ethylene production, conditions possibly lacking in intact seeds (Bewley and Black 1985). *Pinus* seeds, including those from *P. monticola*, often germinate after mechanical disruption of seed coats, especially papery-layers. Ethylene activity varies by species (Ketring 1977) and can be either inhibited or promoted by carbon dioxide or oxygen, and actions of each gas are relatively independent indicating separate sites of action for both gases (Esashi and Leopold 1969). Although seed coat and papery-layer disruption may improve gas exchange allowing for ethylene production, mechanical agitations also stimulate ethylene production (Jaffe 1980).

SUMMARY

Pinus seeds have primary dormancy. *Pinus* embryos in dispersed seeds are mature. Stratification is usually required before *Pinus* seeds germinate. Dormancy in *P. monticola* seeds varies by anatomical site (seed coat, papery-layer, megagametophyte-embryo). Papery-layer induced dormancy is the remaining dormancy after 90-120 days stratification and may limit gas or water exchange. Plant growth regulators, with the possible exclusion of ethylene, have little role in *Pinus* seed germination.

Exogenous ethylene increases germination of some conifer species and the effect of ethylene is increased by warm temperatures. Warm temperature treatments increase germination of *Pinus* seeds, including *P. monticola*. IAA levels in seeds increase during hydration and IAA stimulates ethylene production. This review indicates that possible interactions between the papery-layer, warm treatments, and ethylene contribute to the initiation of germination of *P. monticola* seeds.

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CHAPTER II

PINUS MONTICOLA SEED GERMINATION TREATMENTS

A Review of the Literature

Larsen (1922) called *Pinus monticola* Dougl. ex. D. Don seeds "rebellious" because of their delayed germination. Researchers have attempted to improve *P. monticola* seed germination for nursery production since the early 1900's, but efforts have had little success in promoting rapid, total germination every year. A brief review and evaluation of germination techniques for this species are provided here.

Early work with *P. monticola* reproduction found seeds remain dormant in forest floor (duff) for up to six years (Hofmann 1917). Less than 1% of seeds laying in duff germinated during the first season, whereas most germinated the second year (Haig 1933). Early nursery operators found *P. monticola* seeds had delayed germination for two and three years. Wahlenberg (1924a, 1924b, 1924c) recommended fall sowing to reduce delayed germination. Fall sowing decreased, but failed to eliminate, the nursery manager's seed germination problem.

Early researchers (Rogers 1917; Larsen 1922, 1925) believed *P. monticola* seed germination was impeded by hard seed coats that resisted water and oxygen entry or mechanically restrained primary root egress. Rogers (1917) and Larsen (1925) attempted to improve speed of germination and total germination with a variety of acid and chemical soaks, but these methods lacked stratification (cold-moist after-ripening treatment) and failed to improve germination. Larsen (1925) tried natural stratification in a variety of soils and organic material, chemical and mechanical scarification, and exposure to freezing temperatures with little improvement in germination.

Total germination of *P. monticola* seeds was increased by subjecting seeds to a quick burst of infrared radiation (IR)(Works and Boyd 1964, 1972). IR exposures ranged from 1.56 to 2.47 sec. Fall sown, IR-treated seeds had higher total germination than spring sown seeds stratified 90 days, but total germination was only 35%.

Anderson and Wilson (1966) treated *P. monticola* seeds to various combinations of pre-stratification warm-moist incubations and stratification. A 90-day stratification resulted in the highest speed of germination (10 days), but a 30-day warm-moist incubation (24°C) followed by 30 days stratification (3-5°C) gave the highest total germination (57%), reached in 13 days. The combination of warm-moist and stratification resulted in germination that was nearly double the 90-day stratification percentage. Anderson and Wilson (1966) concluded that a warm after-ripening treatment may be needed.

Nelson (1978) treated seeds with four different water soak combinations followed by 120 days stratification, and one acid scarification treatment. Optimum germination (88-96%) was achieved after a 48-h water soak in which water was initially 49°C. At Potlatch Corporation, this method was only occasionally successful (≥ 90% germination)(R.M. Schaefer III, personal communication).

Procedures for stratifying seeds that usually resulted in ≥ 80% germination were defined by Kidd (1981, 1982). Seeds were soaked 48 h in cold water with oxygen continuously bubbling through the water, then stratified 130 days with a maximum of 1.8 kg of seeds per bag. All seeds were re-treated with oxygen after 43 to 86 days of stratification for 15 min, gently mixed twice each week to ensure good aeration, and checked often to ensure they were moist. This process reportedly yielded 80% germination in eight days.

The University of Idaho method (UI) uses a solution of two parts bleach (5.25% sodium hypochlorite) and three parts water in which seeds are soaked 10 min (Partridge et al. 1985). Bleach-soaking was followed by a 96-h running water wash and cold stratification (2-3°C) for 45 to 50 days. In 1986, Advincula et al. (unpublished data) found the UI method increased germination an average of 14%. The University of Idaho Research Nursery used the UI method but total germination varied from year to

year. Increasing stratification time to 100-150 days helped increase total germination to 80-85% (personal observations).

Malone (1983) obtained higher seed germination when seed coats and papery-layers were removed. Treatment of intact seeds with low concentrations of hydrogen peroxide or 25 days stratification failed to improve total germination (Malone 1983). In addition, Malone (1985) reported on four seed treatments, each beginning with a 48-h running water soak followed by 100 days stratification (1-2°C). In the first treatment, *P. monticola* seeds were placed in a mesh bag and stratified between two layers of peat moss. Every three weeks, seeds were removed and remoistened to maintain a surface film of water on seeds. In the second treatment, soaked seeds were placed in a plastic bag and resoaked 1 h in running water every week. Sowing seeds directly into containers filled with 1:1 (v/v) peat:vermiculite and enclosing the tray in a plastic bag was the third method. The final method was the UI technique (Partridge et al. 1985). Stratification in individual containers yielded the highest total germination (90%), followed by the UI method (80%), stratification in plastic bags (67%), and finally peat stratified (52%)(Malone 1985). The superior total germination of container-treated seeds may result from the individual environment provided each seed. If water-soluble inhibitors were within seeds, this method would preclude them from affecting other seeds, and oxygen concentrations are higher around individual seeds than those in bags (Malone 1985). Unfortunately, the container method requires considerable cooler space and may not work in a bareroot operation because of vagaries of temperature and moisture.

Two stratification techniques were compared by Danielson (1986) where seeds were either presoaked in running water 48 h or left dry before stratification for 56 or 91 days at 3°C. Seeds were stratified on top of peat:vermiculite or between layers of peat moss. Soaking seeds before stratification increased both total germination and speed of germination in the laboratory, but not under field conditions (Danielson 1986). Seeds stratified on peat:vermiculite and seeds stratified between peat layers had similar total germination, results different than those of Malone (1985).

After 105 days stratification, Hoff (1986, 1987) found a procedure for cutting through the seed coat and papery-layers of seeds to expose megagametophyte (referred to as clipping) resulted in prompt germination of viable seeds. Clipping yielded moderate germination even without stratification. Hoff (1987) attributed levels of dormancy to the seed coat (9%), papery-layers (34%) and physiological elements (50%) of the embryo, megagametophyte or both, and the percentage of dormancy contributed by each changed over time. Also, family heritability, an estimate of total variation due to genetic factors, was high (0.83) indicating family genetics strongly influenced dormancy. Physiological dormancy is overcome by 90 days stratification, possibly soon after 42 days (Hoff 1987). Outer seed coat dormancy is removed by 21 days stratification. Papery-layers are the primary element of dormancy restricting germination after 90 and 105 days. These layers may not be a physical impediment to enlarging embryonic tissues but a barrier to water or gas exchange.

Pitel and Wang (1985) treated *P. monticola* seeds with one or more of the following: hydrogen peroxide, kinetin, gibberellic acid, sulfuric acid, and clipping. Moderate increases in germination of nonstratified seeds were obtained by the various treatments, with sulfuric acid soak yielding 42% germination. Seeds stratified 42 days at 4°C and then clipped averaged 68% germination. Seeds stratified 42 days at 4°C, soaked in a solution of 1 mg/L kinetin (6-furfurylaminopurine) and 500 mg/L gibberellic acid (GA_3) followed by a 1.5-h, 35% (w/v) hydrogen peroxide soak, and germinated at cool, constant temperatures of 18-20°C yielded the highest germination (69-83%).

Using 20 family seedlots and four different seed treatments, Gansel (1986) found the following system to give high total germination: 1) 48-h soak in 1% hydrogen peroxide; 2) surface dry; 3) 30 days warm-moist treatment at 10°C; 4) dry to 30-35% moisture content followed by a 90-day stratification at 1-3°C. In Gansel's (1986) test, seedlots with the highest total germination following stratification also had high total germination with the warm/cold method (94% germination in 41 days). Seedlots with low total germination following stratification (≤44%) had 93% germination in 41 days

after receiving the warm/cold method. Most container nursery managers prefer high germination speed but this warm/cold method, when examining all 20 seedlots combined, yielded just 66% germination at 24 days.

Kolotelo (1995) reports the standard Canadian pre-sowing seed treatment included a 48-h soak in running water, after which free-water was drained. Seeds were given a 30-day warm-moist treatment at 20°C, followed by 30-60 days at 3°C. New recommendations are a 48-h soak followed by 12 days in standing water with a daily water exchange during weekdays, followed by a 98-day stratification. Kolotelo (1995) reports the new recommendation increased average total germination an average of 21%.

SUMMARY

Researchers still seek an after-ripening procedure that dissipates the problem of delayed germination of *P. monticola* seeds and is efficient in an operational nursery. Stratification reduces the problem of delayed germination, but effects of the papery-layer continue to delay germination after 90-120 days stratification. Adding a warm treatment (warm water soak, infrared exposure, warm-moist before stratification) often increases total germination over stratification alone. Clipping always increases seed germination and often results in all seeds germinating after 42 days stratification,. For *P. monticola*, much variation in the duration of optimum seed soaking before stratification is reported. *Pinus monticola* seed germination appears to be affected by interactions of the papery-layer, warm conditions, and ethylene, as well as soaking duration.

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CHAPTER III

GERMINATION-ENHANCING TECHNIQUES FOR *PINUS MONTICOLA* SEEDS

INTRODUCTION

He therefore that undertakes the nursery, should be knowing not only in the choice of seeds, where, when and how to sow them; but to know what time of gestation they require in the womb of mother-earth. . .

John Evelyn, Forester, 1664

Pinus monticola Dougl. ex D. Don is one of the most desired reforestation species in the Intermountain West, but its survival and growth is hindered by *Cronartium ribicola* Fischer (blister rust) infection. Breeding efforts resulted in seed orchards yielding progeny with various levels of resistance to blister rust (Howe and Smith 1994). High value orchard seed crops, like other *P. monticola* seed sources, are usually 95% or more viable, but seedling production is limited by poor total germination and speed of germination (Dumroese and Wenny 1987). Researchers have tried various techniques to improve germination. A seed treatment method that yields consistent, high, total germination of high value, improved seeds would increase nursery efficiency, and ensure all genetic potential supplied to the nursery manager is realized in the produced crop. Currently, cultural practices in container and bareroot nurseries are probably reducing genetic diversity of bulk lots of orchard seeds because total germination varies significantly by family (Hoff 1987). Workers removing multiple germinates from containers may favor seedlings from promptly-germinating families that have grown larger than slower-germinating families (Mexal and Fisher 1987; Thomson and El-Kassaby 1993). In bareroot beds, families germinating late during the first growing season or not until the second growing season will not make grade during packing (Mexal and Fisher 1987).

A physiological-based system of defining dormancy was proposed by Lang et al. (1987), with prefixes that describe whether dormancy is induced by the environment (*eco*dormancy), a physical or biochemical factor external to the embryo (*para*dormancy), or physiological factors within the embryo (*endo*dormancy). Factors controlling dissipation of dormancy (i.e. cold temperatures (cryogenic), gas exchange (atmospheric), water (hydrational)) can be added to clarify the type of dormancy. Dormancy sources in *P. monticola* seeds include the papery-layer between the seed coat and megagametophyte and the megagametophyte-embryo (Hoff 1987), but physiological processes that regulate dormancy are unknown. The degree of dormancy found in these anatomical sources changed during stratification (cold-moist treatment), but after 90 days, papery-layers remained the major dormancy source (Hoff 1987). Papery-layers and seed coats of *Pinus* restrict water entry (Baron 1978; Taylor and Wareing 1979; Tillman-Sutela and Kauppi 1995) or limit gas exchange (Stone 1957; Kozlowski and Gentile 1959; Pitel and Wang 1985).

Several techniques increase germination over that achieved with 90 days stratification. Mechanical disruption (clipping) of seed coats and papery-layers to expose megagametophyte tissue consistently increases germination (Pitel and Wang 1985; Leadem 1986; Hoff 1987). A long, warm-moist treatment before stratification increased *P. monticola* seed germination (Anderson and Wilson 1966), and post-stratification warm treatments increased seed germination of *P. strobus* L. (Downie and Bergstem 1991). Another post-stratification treatment, accelerated aging, exposes seeds to very warm (35-45°C) temperatures and 100% humidity for a variety of durations (AOSA 1983). One effect of short, accelerated aging treatment times is increased germination of seeds from southern U.S. *Pinus* spp. (Bonner 1984). Apparently, degradation of polymeric storage compounds and increased seed hydration during accelerated aging causes germination to proceed (Bonner 1984; Chaisurisri et al. 1993). Both pre- and post-stratification warming treatments may also increase ethylene production within seeds (Ketring and Morgan 1970), enhance sensitivity to ethylene (Esashi and Leopold 1969),

and increase phenolic activity (Prasad and Cline 1987), all characteristics associated with seed germination.

Exogenous ethylene applications stimulate conifer seed germination (Hamilton 1972; Borno and Taylor 1975; Shibakusa 1980). Ethylene production is increased by increasing levels of indoleacetic acid (Lieberman 1979). The amount of indoleacetic acid in *P. sylvestris* L. seeds increases sharply during hydration (Tillberg 1977). Seed hydration in *Pinus* follows a three-phase pattern characterized by a rapid initial entry, followed by slow entry, and then another rapid influx immediately proceeding primary root emergence from seeds (Stone 1957; Stanley 1958). Soaking durations to hydrate *P. monticola* seeds vary, ranging from 24 h (Hoff 1987) to 96 h (Partridge et al. 1985); most researchers recommend a 48-h duration (Malone 1985; Danielson 1986; Leadem 1993).

To develop an improved understanding of *P. monticola* seed dormancy and increase germination, my objectives were to 1) examine the effects of soaking duration during phase one seed hydration; 2) determine if air exchange limits germination and define temporal changes, if any, when such limitations occur; 3) compare clipping with warm-moist treatment before stratification; 4) evaluate the post-stratification accelerated aging technique for *P. monticola*; 5) evaluate the role of ethylene in germination; and 6) compare germination when various portions of the embryo-covering layers are removed.

MATERIALS AND METHODS

Four *P. monticola* seed source locations were used for various portions of this study. The first source, Potlatch Corporation 80-01, was collected in 1980 near Elk River, Idaho (latitude 46°46' N, longitude 116°9'W) at 900 m elevation. The second source, Plum Creek Timber Company 21-1352 (PCTC), was collected in 1982 from northeastern Washington (latitude 48°35' N, longitude 117°35'W) at 1070 m elevation. Because large amounts of seed were necessary to meet experimental needs, I used seeds collected from the Moscow Arboretum (MA)(latitude 46°45', longitude 117°00') at 700 m elevation, in five different years (1990, 1992-1995). The fourth seed source, Hoff Collection (HC) was from the Palouse River drainage, Idaho (latitude 46°59' N, longitude 116°33'W) at 823 m elevation. Cones were collected from 20 individual trees (families) in 1982 (see Hoff 1987). Total germination after 90-105 days stratification varied greatly between HC families. All seeds, after extraction from cones and drying to 6-8% moisture content, were freezer-stored (-15°C) until used.

Unless specified, seeds in each replicate were soaked 48 h in running tap water (13°C) before stratification. Stratification consisted of spreading seeds thinly onto a moistened Kimpak (Type K-22, 12-ply; Kimberly-Clark, Neenah, Wisconsin, USA), covering them with another moist Kimpak, and carefully placing the sandwich into unsealed plastic zip-lock-type bags (17.8 cm x 20.3 cm) for stratification at 1-2°C. For most experiments involving clipped seeds, after the designated stratification duration, I used Hoff and Steinhoff's (1986) technique of cutting through the seed coat and papery layer to expose the megagametophyte (Fig. 3.1.B). After stratification or post-stratification treatments, seeds were incubated in Wang polycarbonate germination boxes (Spencer-Lemaire Industries, Ltd., Edmonton, Alberta, Canada) on moistened Kimpaks at 20-22°C. A count of germinated seeds was made at 7, 14, 21, and 28 days. Seeds with primary roots extending at least half the seed coat length were considered germinated.

Counts of germinated seeds in the six germination experiments and seed moisture content experiment were analyzed by ANOVA using PROC GLM, and the correlation between seed moisture content and germination was determined using PROC REG in SAS (SAS Institute Inc. 1989, 1993). Residuals were plotted and their distribution was normal, independent, and homogenous, making data transformation unnecessary. For ANOVA's on each seed source and the ANOVA over seed sources, means at each stratification or soaking duration within an experiment were compared using Tukey's studentized test at the 0.05 level of significance.

Exposed Megagametophyte Placement

In this experiment, germination of clipped seeds from HC 15 and 31 with exposed megagametophytes placed against or away from a moist substrate was compared. After each of five stratification durations (0, 21, 42, 90, or 120 days), 120 seeds per family were clipped with a modified Hoff and Steinhoff (1986) technique so the cut surface was higher on the seeds (Fig. 3.1.D). Half the clipped seeds were divided into three replicates of 20 seeds (60 seeds/family) and placed so the exposed megagametophyte touched a moist Kimpak. The remaining 60 seeds/family were divided into three 20 seed replicates and exposed megagametophytes were placed away from the moist Kimpak toward air.

Warm-moist Treatment vs. Clipping

Seedlots PCTC and 80-01 were used to examine interactions between a warm-moist treatment before stratification and clipping. Data provided by Plum Creek Timber Company and Potlatch Corporation showed these lots, with 120 days stratification, germinated at about 5% (PCTC) and 10% (80-01). From each seedlot, 720 seeds were soaked and then divided into four treatments: control (stratification only), clipped (Fig. 3.1.B), warm, and warm/clipped (Fig. 3.1.B). Each treatment was replicated three times (20 seeds/replicate) per family at each of three stratification durations (0, 45, or 90 days). For the warm treatment, after soaking, seeds were spread thinly onto a moist Kimpak, left uncovered, and placed into a 1.5 mil-thick plastic bag for 10 days at 20-24°C. Each day, the bag was opened 30 min to allow air circulation. After 10 days, seeds were covered with another moist Kimpak and stratified. Seeds receiving the warm/clipped treatment were given the same warm-moist treatment and stratification as warm-treated only seeds, but were clipped before placement in germination boxes.

Accelerated Aging

I used seeds from 80-01 and MA 1990 and 1992 to investigate accelerated aging on germination and to compare accelerated aging with clipping. Because all other freezer-stored 80-01 seeds had been used, I used seeds stored seven years in a refrigerator (3-5°C) for this experiment. From each of the three seedlots, 2400 seeds were divided into four treatments: control (stratification only), clipped (Fig. 3.1.B), and 24- or 48-h accelerated aging. Each treatment was replicated four times with 50 seeds at each of three stratification durations (0, 45, or 90 days). After stratification, seeds were placed in a Stults accelerated aging chamber and exposed to 40°C and 100% humidity (AOSA 1983) for 24 or 48 h before placement in germination boxes.

Ethylene

To assess a possible role of ethylene in germination, I used HC 7, 10, and 30, and 80-01. From each seedlot, 1440 seeds were divided into eight treatments including control, warm, clipped (Figure 3.1.B), two ethylene precursors (Kelpak 66 or ethephon), and three ethylene inhibitors (cobalt nitrate, silver nitrate or silver thiosulfate). Kelpak 66 is commercially-extracted from *Ecklonia maxima* (Osbeck) Papenfuss. Ethephon is (2-chloroethyl) phosphonic acid. Control, warm-treated, and clipped seeds were treated as described above, except that seeds were soaked 48 h in tap water changed every 12 h. Seeds destined for treatment with either ethylene inhibitors or precursors were placed into mesh bags before soaking 48 h in appropriate solutions described below. Each solution was changed every 12 h. At each of three stratification durations (0, 45, or 90 days), three replicates of 20 seeds per seedlot and treatment were germinated as described above.

Kelpak 66 was diluted 1:500 (v/v) in distilled water as recommended by the manufacturer (Arbor Grove, Inc.; Fairfield, NJ). A 2 mM silver nitrate ($AgNO_3$) solution was prepared mixing 339.8 mg

$AgNO_3$ in one liter distilled water and silver thiosulfate was prepared by mixing equal volumes of 2 mM $AgNO_3$ and 8 mM sodium thiosulfate ($Na_2S_2O_3 \cdot 5\ H_2O$)(Veen and van de Geijn 1978). Based on work by Miller and Roberts (1984), ethephon solution was prepared by diluting 0.1 ml (2-chloroethyl) phosphonic acid in one liter distilled water, and an 8mM cobalt nitrate ($Co(NO_3)_2$) solution (4x the molarity of $AgNO_3$) was made using 2.927 g/L. All solutions were refrigerated (3°C) in the dark until used.

Seed Moisture Contents and Resulting Germination

To determine the moisture content of freezer-stored seeds, 10 replicates of 100 seeds from each of MA 1993, 1994, and 1995 were weighed, oven-dried 24 h at 60°C, reweighed, and oven-dried an additional 24 h before a final weight was measured. To determine the effects of soaking duration on germination of intact and clipped seeds, seeds from MA 1993, 1994, and 1995 were assigned to one of two treatments: clipped at the micropylar end (Fig. 3.1.C) or left intact (1000 seeds/seedlot/treatment). For each family, 10 replicates of 100 seeds from each treatment were soaked 120 h in 20°C water changed every 12 h. At 24-h increments, seeds, a replicate at a time, were blotted dry, weighed, and returned to soak. After 120 h, seeds were oven-dried 24 h at 60°C. To assess an adequate soaking duration, four replicates of 50 intact seeds from the same seedlots were soaked for one of five durations (24, 48, 72, 96, or 120 h) in running tap water (13°C)(1000 total seeds/seedlot). After soaking, seeds were blotted dry, reweighed, sealed into zip-lock-type plastic freezer bags (17.8 cm x 20.3 cm), and stratified 90 days at 1-2°C. After stratification, seeds were reweighed and germinated as described above. Moisture contents were calculated by dividing the difference between wet weight and oven-dry weight by oven-dry weight and multiplying by 100.

Excised Embryos

Nonstratified MA 1993, 1994, and 1995 seeds were surface sterilized in 40% bleach (5.25% sodium hypochlorite) for 10 min, and washed 6 h in sterile water. From each seedlot, 288 sterile seeds were assigned to one of four treatments in which the following layers covering the embryo were removed: nondissected (intact) (control), seed coat (Fig. 3.1.E), papery-layer, and megagametophyte. Each treatment was replicated twice with 36 seeds per replicate. Explants were cultured on ½ Gresshoff and Doy (1972) medium and were maintained at ≈24°C with an 18-h photoperiod at 1500 lux. Seeds whose primary roots grew ≥ 2 mm were considered germinated when counts were taken after 1, 4, and 18 weeks.

Scanning Electron Microscopy

Forty seeds from HC 10, 25, 39, and 48 were examined by a scanning electron microscopy to determine visual differences between papery-layers after stratification durations of 0, 21, 42, or 90 days. Intact seeds and seeds with outer testae removed (papery-layers mostly intact; Fig. 3.1.E) were placed into stainless steel histological cages (5 seeds per cage/seed treatment/family/stratification duration). Family selection was based on different total germination of intact seeds (Hoff 1987; Hoff, unpublished data). Levels of papery-layer dormancy were low (HC 39), moderate (HC 10), and high (HC 25 and 48)(Hoff 1987; Hoff, unpublished data). Cages containing seeds were immersed in a fixation solution of 2.5% gluteraldehyde in a 0.1 M cacodylate buffer. After soaking 1 h, seeds were rinsed 5 min each in three baths of 0.1 M cacodylate buffer. Seeds were also fixed 1 h in 2.0% osmium tetroxide diluted 1:1 with distilled water. I rinsed seeds 5 min in each of four distilled water baths. Seeds were dehydrated in five steps, each lasting 10 min, using progressively more concentrated (30, 50, 70, 95, and 100%) ethyl alcohol. After treatment in a critical-point drier, intact seeds were gripped

with two pairs of locking forceps, dipped in liquid nitrogen for 5 sec, and broken in half. Seeds with testae removed and broken seeds were mounted on aluminum stubs and gold coated for 20 sec using a Technics Hummer Sputter Coater V. Prepared seeds were viewed with a Hitachi S-570 at 20 kv at magnifications of 70-1300x.

RESULTS

Seed germination was influenced by a three-way interaction in each experiment indicating germination varied between combinations of seed source, treatment, and stratification duration. An interaction was expected since variations in total germination were a criterion for selecting seed sources for each experiment. Because the pattern of germination over stratification duration was similar for seed sources, only total germination varied at specific stratification durations. My results will focus on two-way interactions between treatment and stratification duration.

Germination increased, regardless of treatment, with increasing stratification duration (Figs. 3.3-3.6 and 3.8). When clipped seeds germinated, speed of germination was rapid. Germination of clipped seeds was often observable after 24 h and 75% of the seeds germinated within seven days after being placed into germination trays. In contrast, germination of intact seeds was slower; 75% of total germination was reached at 21 days.

Exposed Megagametophyte Placement

Seeds had portions of seed coats and papery-layers removed to expose megagametophyte (Fig. 3.1.D) after stratifying for various durations. Exposed megagametophytes were placed against a moist germination substrate or away from moist substrate. When stratification durations were less than 90 days, more seeds with exposed megagametophytes placed away from moist substrate germinated than seeds with exposed megagametophytes placed against moist substrate (Fig. 3.3). The improvement in total germination dropped from 27% to 7% between zero and 90 days stratification. Total germination was similar after 120 days stratification regardless of megagametophyte exposure.

Warm-moist Stratification vs. Clipping

Seeds were given a warm-moist treatment before stratification, or clipped after stratification (Fig. 3.1.B), or given both treatments, for various stratification durations. Nonstratified seeds failed to germinate (Fig. 3.4). Germination of seeds clipped or warm-moist treated and clipped was similar at all stratification durations. By 90 days stratification, the warm-moist treatment before stratification and the clipping treatment yielded similar total seed germination.

Accelerated Aging

Germination of seeds exposed to high temperature and humidity after various stratification durations was compared with clipping (Fig. 3.1.B) and stratification. More seeds treated with accelerated aging for 48 h germinated than stratified-only seeds, if all seeds were provided 45 days stratification (Fig. 3.5). About 60% more clipped seeds germinated than control seeds and exceeded total seed germination of accelerated aging treatments by at least 32% after 45 days stratification. Shorter exposure to accelerated aging conditions (24 h) failed to affect germination. Germination yield by all treatments was similar after 135 days stratification.

Ethylene

Germination of seeds soaked in ethylene precursors or inhibitors was compared to germination of seeds clipped after stratification (Fig. 3.1.B), and to germination of seeds given a warm-moist treatment before stratification. Soaking seeds before stratification in ethylene precursors or inhibitors failed to improve total germination when compared to the nonstratified control (Table 3.1). Warm-moist treatment and clipping yielded 40% and 30% more seed germination, respectively, than the other treatments after 90 days stratification. Without stratification, clipping resulted in 27% germination, whereas seeds receiving other treatments failed to germinate. Silver thiosulfate yielded the same total seed germination as clipping, although it was also statistically similar to the control. The silver nitrate treatment caused 100% seed mortality as evidenced by dark, soggy megagametophytes and embryos.

Seed Moisture Contents and Resulting Germination

Intact seeds were soaked 120 h in 13°C running water. Increasing soaking duration increased seed moisture content (Fig. 3.6.A). One day of soaking resulted in the lowest seed moisture contents after soaking and after stratification (Fig. 3.6.B), and subsequent total germination was also the lowest. A 72-120 h soaking duration yielded the highest post-stratification seed moisture contents and the highest total germination (Fig. 3.6.C). The r^2 for soaking duration on germination was 0.7. A better predictor of germination was achieved with soaking duration and either post-soaking or post-stratification moisture content (r^2=0.8).

Moisture content of seeds clipped at the micropyle (Fig. 3.1.C) was compared to moisture content of intact seeds soaked 120 h in 20°C water changed every 24 h. Clipping resulted in ≈20% higher seed moisture contents than those in intact seeds at every soaking duration (Fig. 3.7). Intact seeds with longer soaking durations contained significantly more water, with seeds receiving the 96 and 120 h durations having 52% moisture contents. For all seedlots and soaking durations combined, clipping resulted in 57% moisture content compared with 50% for intact seeds. The shortest soaking duration (24 h) with clipped seeds resulted in as high a moisture content as the longest duration (120 h) with intact seeds. For intact seeds, the shortest soaking duration at 20°C had similar moisture contents (45%) to the longest soaking durations at 13°C.

Excised Embryos

Germination of nonstratified MA 1993, 1994, and 1995 seeds in vitro, with various embryo-covering layers (seed coat, papery-layer, megagametophyte) removed, was compared. Total germination of excised *P. monticola* embryos was 99% in seven days; remaining embryos were contaminated by fungi introduced during initiation of the experiment (Fig. 3.8). Embryo germination was complete within 10 days. The papery-layer encasing the megagametophyte was torn when seed coats were removed (Fig. 3.1.E). Embryos retained within megagametophytes germinated at half the rate of excised embryos, but intact seeds failed to germinate. After 24 h, excised embryos were swelling and primary roots reddening. After 48 h, primary roots began curving downward, hypocotyls and cotyledons had begun to green and cotyledons were opening. After 18 weeks of culture, 20-47% more of the embryos retained in megagametophytes and one-third of intact seeds germinated.

Scanning Electron Microscopy

Intact seeds and seeds with testae removed but papery-layers mostly intact were examined with scanning electron microscopy. The structure of papery-layers appeared similar among families with low (HC 39), moderate (HC 10), or high (HC 25 and 48) levels of papery-layer dormancy as defined by

Hoff (1987, unpublished data). Large variation, quantified as the number of layers comprising the papery-layer, was noted between seeds within families. For all families at all stratification durations, some seeds had holes in the papery-layer (Fig. 3.2). The number of seeds with holes within families, between families, and at each stratification duration was highly variable.

DISCUSSION

Most nonstratified, hydrated seeds of *P. monticola* fail to germinate under what appear to be favorable conditions; *P. monticola* seeds are dormant. However, my results show excised, nonstratified embryos of *P. monticola* germinate rapidly and grow into seedlings without transfer of sucrose or other metabolites from the megagametophyte. Therefore, *P. monticola* embryos are not endodormant, but rather paradormant. Since dormancy is due to factors outside meristematic tissue of the seed, *P. monticola* seeds are paradormant (c.f. Lang et al. 1987). Although Hoff (1987) concluded that about 50% of dormancy is associated with the physiological condition of the megagametophyte-embryo, my results show paradormancy is fully associated with the megagametophyte. Megagametophyte paradormancy is evident in at least two ways. In vitro, about half of the megagametophyte-encased embryos removed from seeds germinated, indicating seed coats and/or papery-layers restricted gas and/or water exchange (atmospheric and/or hydrational paradormancy). The remaining megagametophyte-encased embryos failed to germinate after 18 weeks at favorable germination temperatures. Therefore, cryogenic paradormancy is exhibited in about half the population of nonstratified, hydrated *P. monticola* seeds.

Clipping seeds, especially when stratification durations were ≤ 45 days, always yielded higher germination than that of intact seeds (Figs. 3.4-3.5). Hoff and Steinhoff's (1986) technique provides seeds access to both water and air. Moisture content in *P. monticola* seeds can be increased by longer soaking durations, soaking at warmer temperatures, and disrupting seed coat integrity, a trait shared by some *Pinus* spp. (Stone 1957; Baron 1978; Qi et al. 1993). Longer soaking durations (72-120 h) improved total germination of *P. monticola* seeds (Fig. 3.6.C), indicating papery-layers may be associated with hydrational paradormancy. The possibility of hydrational paradormancy is consistent with work on *P. lambertiana* Dougl. seeds (Baron 1978) but contrary to that of *P. jeffreyi* Grev. & Balf. seeds (Stone 1957). Water entry is probably limited during early stages of seed hydration (0-72 h), but my data show seed moisture content stabilizes with an additional 24-48 h soak, much faster than that reported during stratification (Stone 1957; Murphy and Noland 1982b). A lack of continued water entry into seeds during the second phase of water entry during stratification explains the poor germination of seeds soaked 24 h in this study (no external water source during stratification), and better results with Hoff's (1987) 24-h soak and moist substrates during stratification. For soaking durations, highest total germination of seeds was achieved by soaking ≥ 72 h and a subsequent moisture content of 35% (Fig. 3.6.B). This seed moisture content was optimum for stratification of *P. strobus* seeds (Downie and Bergstem 1991) and is within the range hypothesized for *P. monticola* seeds (Gansel 1986). Therefore, hydrational paradormancy is not a factor when *P. monticola* seeds are soaked and then stratified in the presence of exogenous moisture. Conversely, my exposed megagametophyte placement data shows more stratified seeds with exposed megagametophytes placed toward air germinated than seeds with exposed megagametophytes oriented toward moisture (Fig. 3.3), indicating gas exchange is limited in intact seeds. This finding is similar to non-stratified *P. monticola* seeds (Pitel and Wang 1985) but contrary to work with stratified *P. lambertiana* seeds (Baron 1978). Therefore, clipping dissipates atmospheric paradormancy within *P. monticola* seeds.

Total germination of warm-treated (warm-moist or accelerated aging) seeds was higher than control seeds when stratification duration was ≤ 45 days (Figs. 3.4-3.5). Seeds hydrated, nonstratified, and exposed to the warm-moist treatment or accelerated aging had less total germination than similarly

treated seeds that were also clipped; total germination of seeds given a warm-moist treatment or accelerated aging was similar to intact, nontreated seeds. Stratified seeds with warm-moist treatment or accelerated aging that were also clipped had higher total germination than those not clipped (Figs.3.4-3.5). Therefore, warm-moist treatment and accelerated aging reduce atmospheric paradormancy but were ineffective on cryogenic paradormancy. After 90 days stratification, more seeds from both the warm-moist treatment and accelerated aging treatments germinated than control seeds, and seeds given the warm-moist treatment had similar total germination to clipping (Fig. 3.5). Therefore, pre-stratification exposure of seeds to the warm-moist treatment appears more beneficial than post-stratification exposure via accelerated aging. The ineffectiveness of the short accelerated aging duration may have been a result of either too much or too little metabolic activity (Blanche et al. 1990) or excessive cellular membrane disruption (Murphy and Noland 1982a).

In the exposed megagametophyte placement experiment, germination of seeds with megagametophytes exposed to air and seeds with megagametophytes exposed to moisture was best described linearly, and the ratio of the slopes of these lines was 0.4. Germination of warm-moist treated seeds, and warm-moist treated seeds with clips was also best described linearly, and the ratio of the slopes of these lines was also 0.4, indicating dissipation of atmospheric dormancy in both experiments is similar. How does clipping and the warm-moist treatment dissipate atmospheric paradormancy?

During stratification, respiration is aerobic and sufficient for the slow accumulation of oxygen-rich metabolic products without considerable use of storage materials (Nikolaeva 1969). Such products, including adenosine triphosphate (ATP), are probably necessary for certain aspects of protein synthesis. Accumulation of oxygen-rich metabolites or ATP may need to reach a critical level, a threshold, before germination occurs. A threshold level was hypothesized by Stone (1957) and is evident in several agronomic crops (Mayer and Poljakoff-Mayber 1989). *Pinus monticola* seeds respire at the same rate whether in stratification or when transferred from stratification to warmer temperatures (Leadem 1993). However, *P. monticola* seeds without papery-layers have a higher potential for respiration (Leadem 1993). I hypothesize that clipped seeds, placed in warm conditions favorable for growth, have largely unimpeded access to oxygen. Therefore, respiration proceeds at peak rates providing high levels of ATP or an oxygen-requiring metabolite and quick attainment of the necessary threshold to evoke germination. Without clipping, seeds continue to accumulate ATP or an oxygen-requiring metabolite, thus slowly decreasing the amount of oxygen needed to reach the threshold. My data, showing the temporal effect of air-directed exposed megagametophytes ameliorated by increasing stratification durations, supports this idea. Warm-moist treatments have been shown to increase the rate of processes like sucrose hydrolysis (Murphy and Hammer 1988), protein synthesis (Noland and Murphy 1986; Murphy and Hammer 1993), enzyme activity (Murphy et al. 1980; Noland and Murphy 1984), and ATP formation (Ching and Ching 1972; Noland and Murphy 1984; Bourgeois and Malek 1991). Thus, warm-moist treatments causing initial surges of ATP or oxygen-requiring metabolite production may effectively lower the amount of ATP or the metabolite needed to accumulate during stratification.

Paradormancy in *P. monticola* seeds can be represented as in Figure 3.9. For intact seeds, total germination reflects the portion of the population without cryogenic or atmospheric paradormancy. When intact seeds are warm-treated before stratification, the total amount of atmospheric paradormancy is reduced. Reduced atmospheric paradormancy lowers the germination-evoking requirement and allows higher germination at each stratification duration. Total germination of clipped seeds can only be as high as the portion of the population without cryogenic paradormancy.

Soaking seeds with ethylene precursors or inhibitors failed to affect total germination in this study, but treating seeds with silver thiosulfate yielded similar germination to the clipping treatment after 45 days stratification. Ethylene formation is an oxygen-requiring process. Blocking ethylene formation with silver thiosulfate may have allowed oxygen to be used for ATP production or caused an accumulation of oxygen-requiring metabolites. Endogenous ethylene generation could be at such a reduced level that allowing the transfer of oxygen from ethylene production to other metabolic activities is not a

particularly significant event. Although I tested several ethylene precursors and inhibitors at concentrations likely to show an effect, testing at a broader range of ethylene precursor and inhibitor concentrations may still be warranted, especially since effective concentration and treatment duration vary by species (Hamilton 1972) and because of the paucity of research relating ethylene effects on conifer seeds, especially *Pinus* species.

CONCLUSIONS

Seeds of *P. monticola* are paradormant. The megagametophyte is the site of cryogenic and atmospheric paradormancy. Cryogenic paradormancy dissipates with stratification duration. Seed coats, including papery-layers, impede water entry during initial seed hydration, but evidence for hydrational paradormancy was lacking. Atmospheric paradormancy is regulated by papery-layers and decreases with increasing stratification duration. Dissipation of atmospheric paradormancy is probably due to an accumulation of metabolites necessary to evoke germination. Exposing seeds to warm-moist conditions before stratification is not detrimental, and such exposure appears to lower the threshold necessary for dissipation of atmospheric paradormancy. Ethylene, in my experiments, appears not to be involved with *P. monticola* seed germination. Germination-enhancing techniques (clipping, warm-moist treatment, accelerated aging) are uninvolved with the dissipation of cryogenic paradormancy; they only facilitate processes required to evoke germination.

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Table 3.1. *Ethylene.* Germination of seeds either clipped, soaked in ethylene inhibitors, soaked in ethylene precursors, warm-moist treated, or stratified only for each stratification duration. Mean total germination for each treatment at each duration is the average total germination of seed sources HC 7, 10, and 30 and 80-01 combined (each seed source had three replicates of 20 seeds).

Treatments	Stratification duration (days)		
	0	45	90
	Germination (%)		
Control (stratified only)[1]	2 a[9]	39 b	58 b
Warm-moist treated[2]	0 a	41 b	96 d
Clipped[3]	27 b	71 c	83 cd
Ethylene Inhibitors:			
Cobalt nitrate[4]	0 a	45 b	57 b
Silver thiosulfate[5]	0 a	48 bc	67 bc
Silver nitrate[6]	0 a	0 a	0 a
Ethylene Precursors:			
Kelpak 66[7]	0 a	38 b	58 b
Ethephon[8]	1 a	27 b	53 b

[1]Soaked 48 h in water changed every 12 h, stratified at 3°C between moist Kimpaks.
[2]Soaked as above, incubated 10 days at 22°C, stratified as above.
[3]Soaked as above, stratified as above, then clipped as described by Hoff and Steinhoff (1986).
[4]Soaked 48 h in 8 mM $Co(NO_3)_2$ changed every 12 h, stratified as above.
[5]Soaked 48 h in 8 mM $Na_2S_2O_3 \cdot H_2O$ changed every 12 h, stratified as above.
[6]Soaked 48 h in 2 mM $AgNO_3$ changed every 12 h, stratified as above. Silver nitrate caused 100% mortality evidenced by dark, soggy megagametophytes and embryos.
[7]Soaked 48 h in 1:500 (v/v) Kelpak 66 changed every 12 h, stratified as above. Kelpak commercially-extracted from *Ecklonia maxima* (Osbeck) Papenfuss, Arbor Grove, Inc., Fairfield, NJ.
[8]Soaked 48 h in solution of 0.1 ml (2-chloroethyl) phosphonic acid in one liter water changed every 12 h, stratified as above.
[9]Means in columns with different letters are different at $p \leq 0.05$ using Tukey's studentized test.

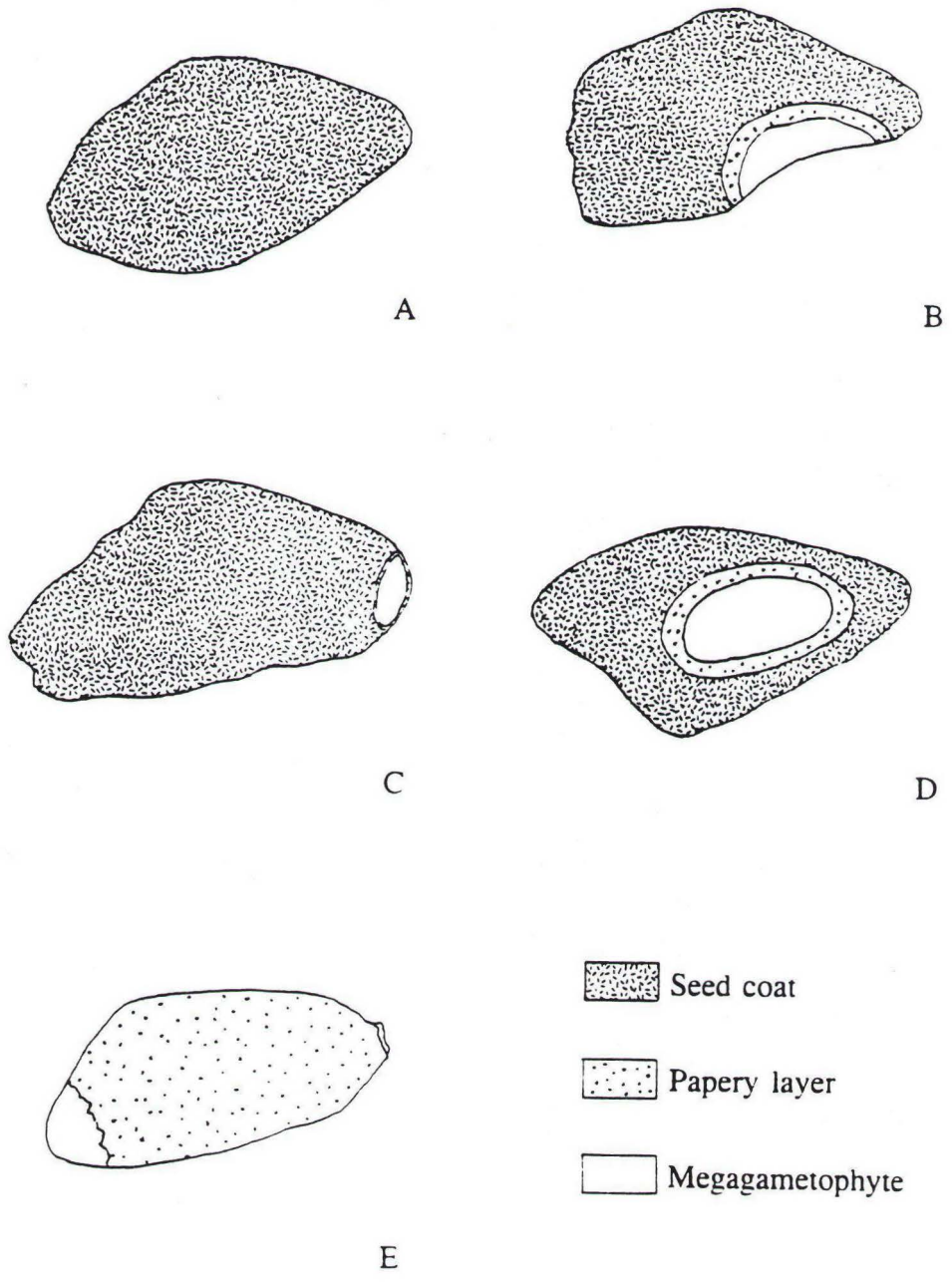

Figure 3.1. Intact seed (A), seed clipped with the Hoff and Steinhoff (1986) technique (B), micropylar cut on a seed (C), seed with the modified clip (D), and seed with seed coat removed (E).

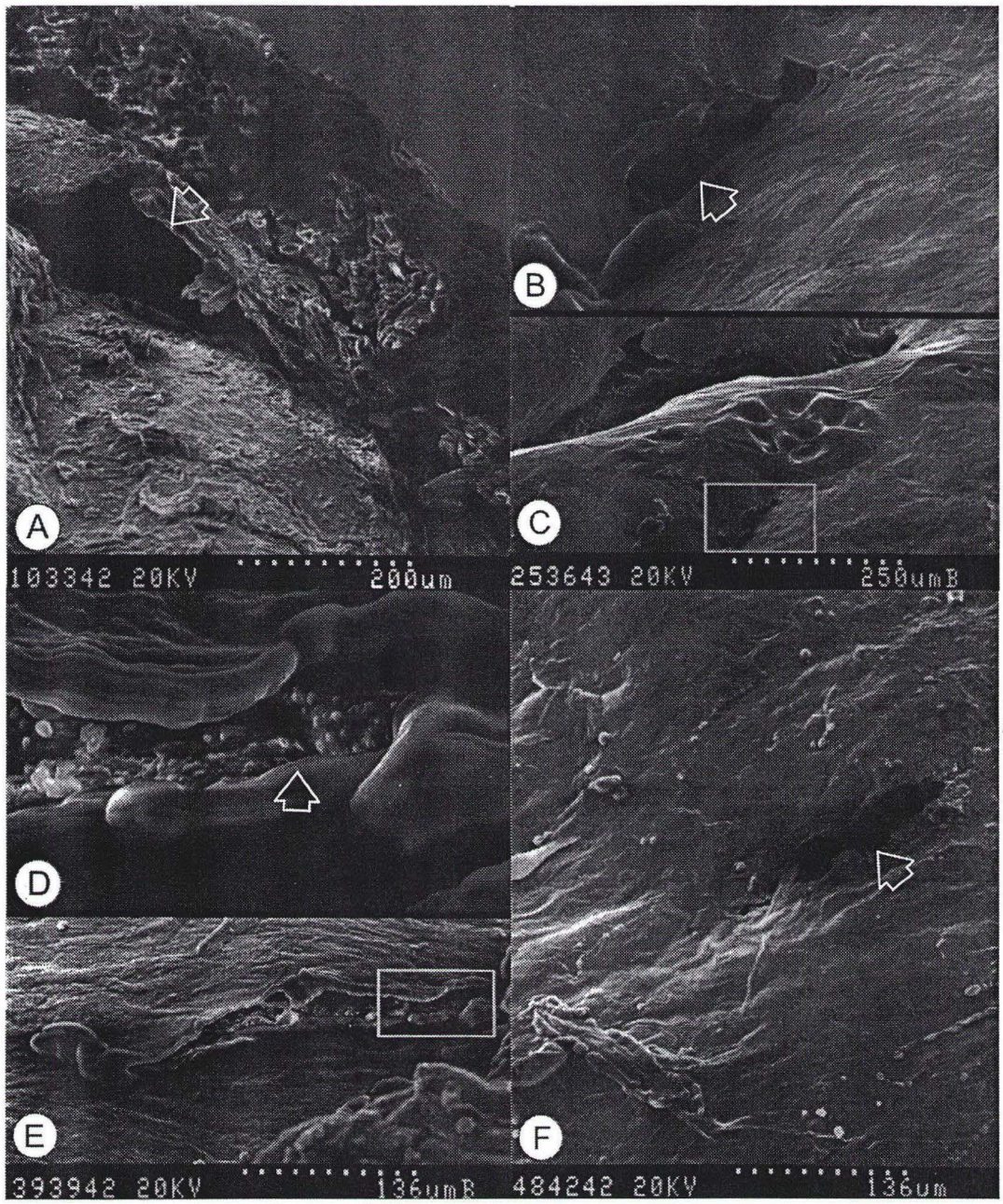

Figure 3.2. Scanning electron micrographs of papery-layers from seeds of HC 10 (A), 25 (B and C), 39 (D and E), and 48 (F) after 42 days stratification, showing holes (arrows) in the papery-layers. Details in white boxes in B and D shown at an additional 5x in C and E, respectively.

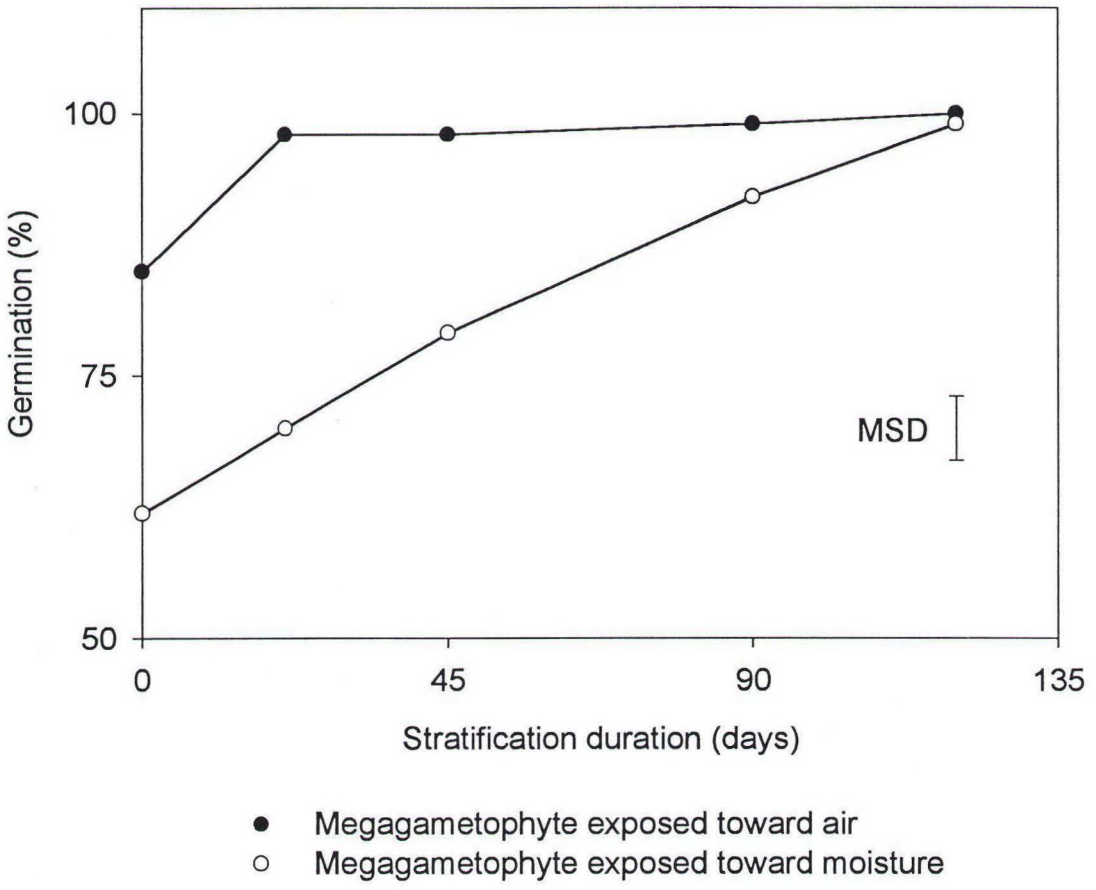

Figure 3.3. *Exposed Megagametophyte Placement.* Average seed germination percentages of HC 15 and 31 (combined) with different clip placement and stratification durations. Each mean is the average germination of two familes each replicated three times with 20 seeds per replicate. Tukey's mean significant difference (MSD) was calculated at the 0.05 level of significance. Using linear regression, the equation for germination of seeds with clips placed toward air was best described by $ß_0 + ß_1$ where $ß_0 = 85.8$ and $ß_1 = 0.16$ ($r^2 = 0.96$). The equation for germination of seeds with clips placed toward moisture was best described by $ß_0 + ß_1$ where $ß_0 = 62.2$ and $ß_1 = 0.38$ ($r^2 = 0.99$). Ratio of the slopes is 0.42.

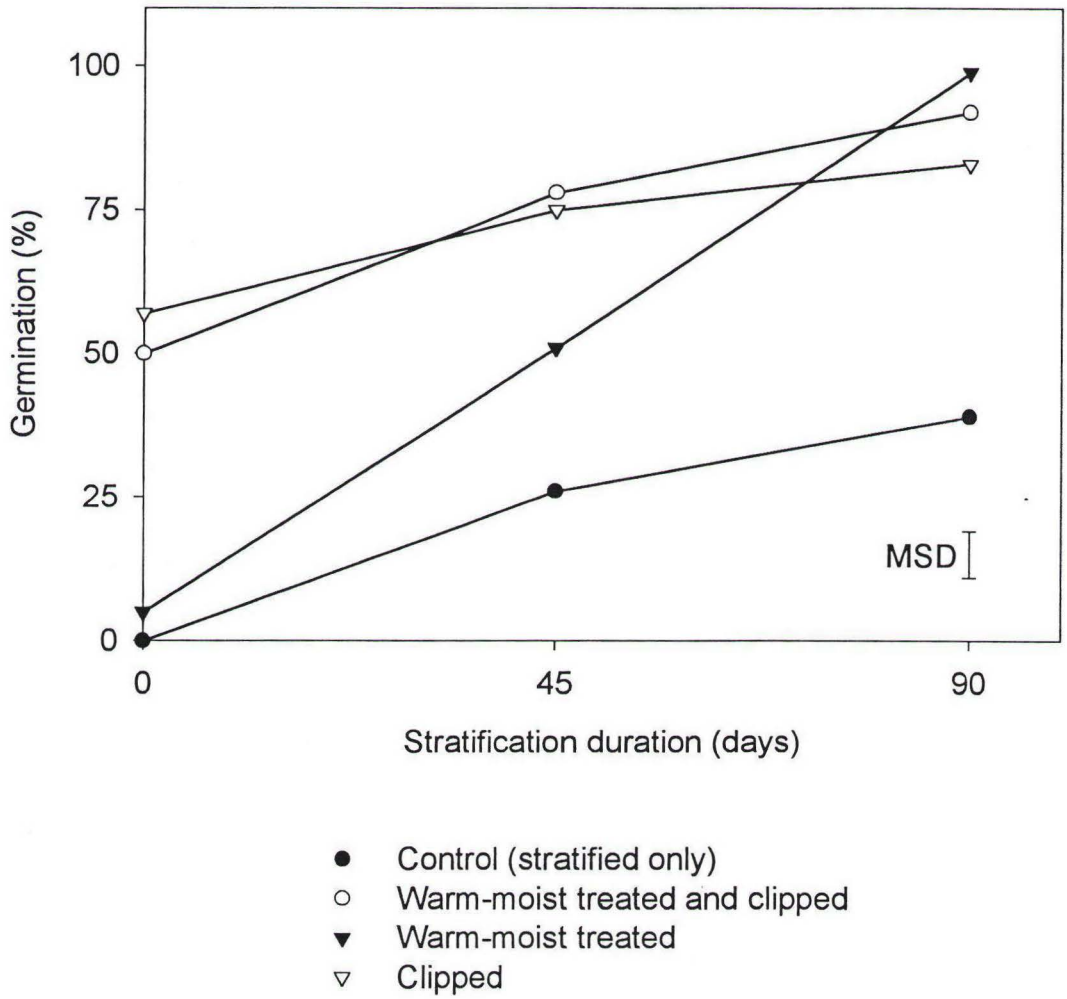

Figure 3.4. *Warm-moist Treatment vs. Clipping.* Average seed germination percentages of PCTC and 80-01 (combined) with different seed treatments and stratification durations. Means are the average germination of two seedlots each replicated three times with 20 seeds per replicate. Tukey's mean significant difference (MSD) was calculated at the 0.05 level of significance. Using linear regression, the equation for germination of seeds with warm-moist treatment and clips was best described by $ß_0 + ß_1$ where $ß_0 = 56.3$ and $ß_1 = 0.42$ ($r^2 = 0.98$) The equation for germination of seeds with warm-moist treatment was best described by $ß_0 + ß_1$ where $ß_0 = 2.16$ and $ß_1 = 0.94$ ($r^2 = 0.99$). Ratio of the slopes is 0.45.

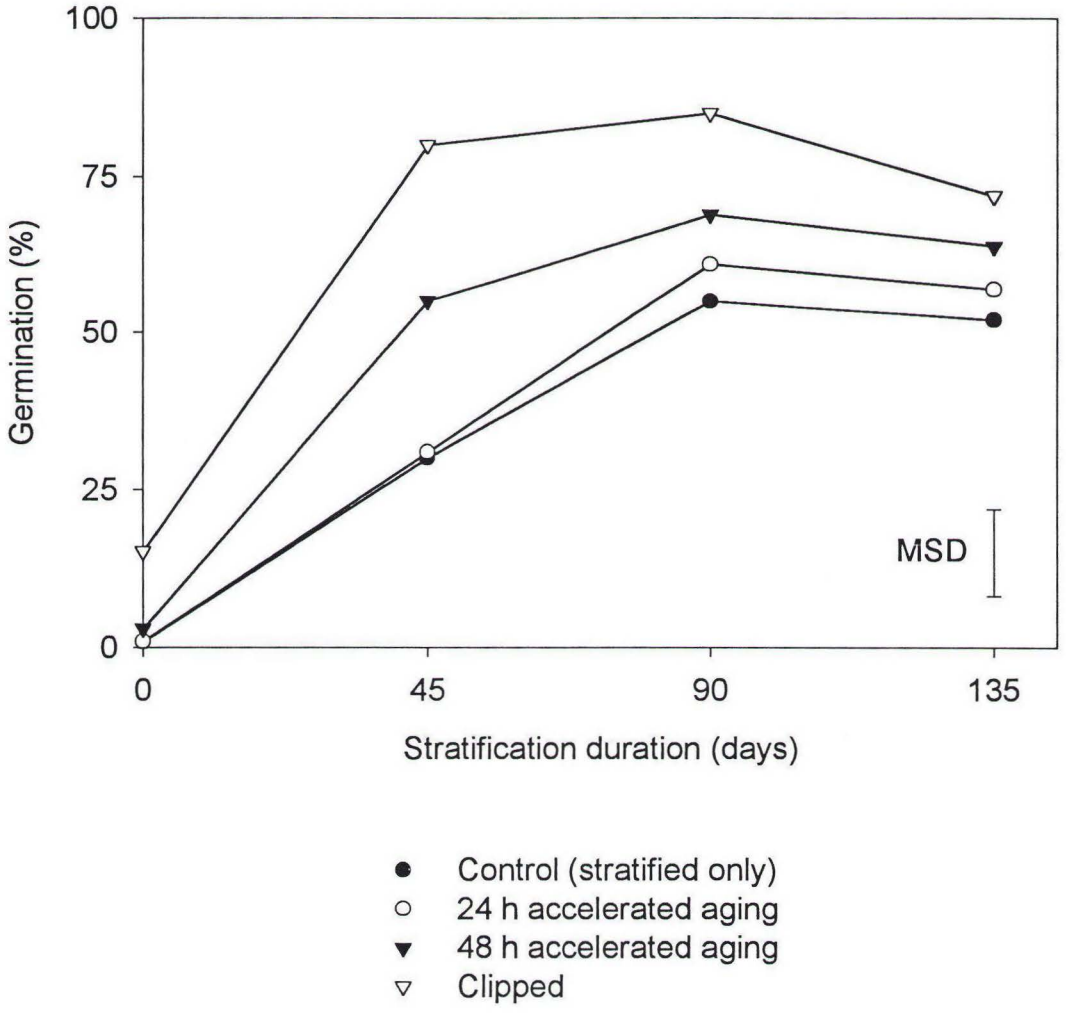

Figure 3.5. *Accelerated Aging.* Average seed germination percentages after 24 or 48 h accelerated aging or clipping for various stratification durations for seedlots MA 1990 and 1992 and 80-01 (combined). Each mean is the average germination of three seedlots each replicated three times with 20 seeds per replicate. Tukey's mean significant difference (MSD) was calculated at the 0.05 level of significance.

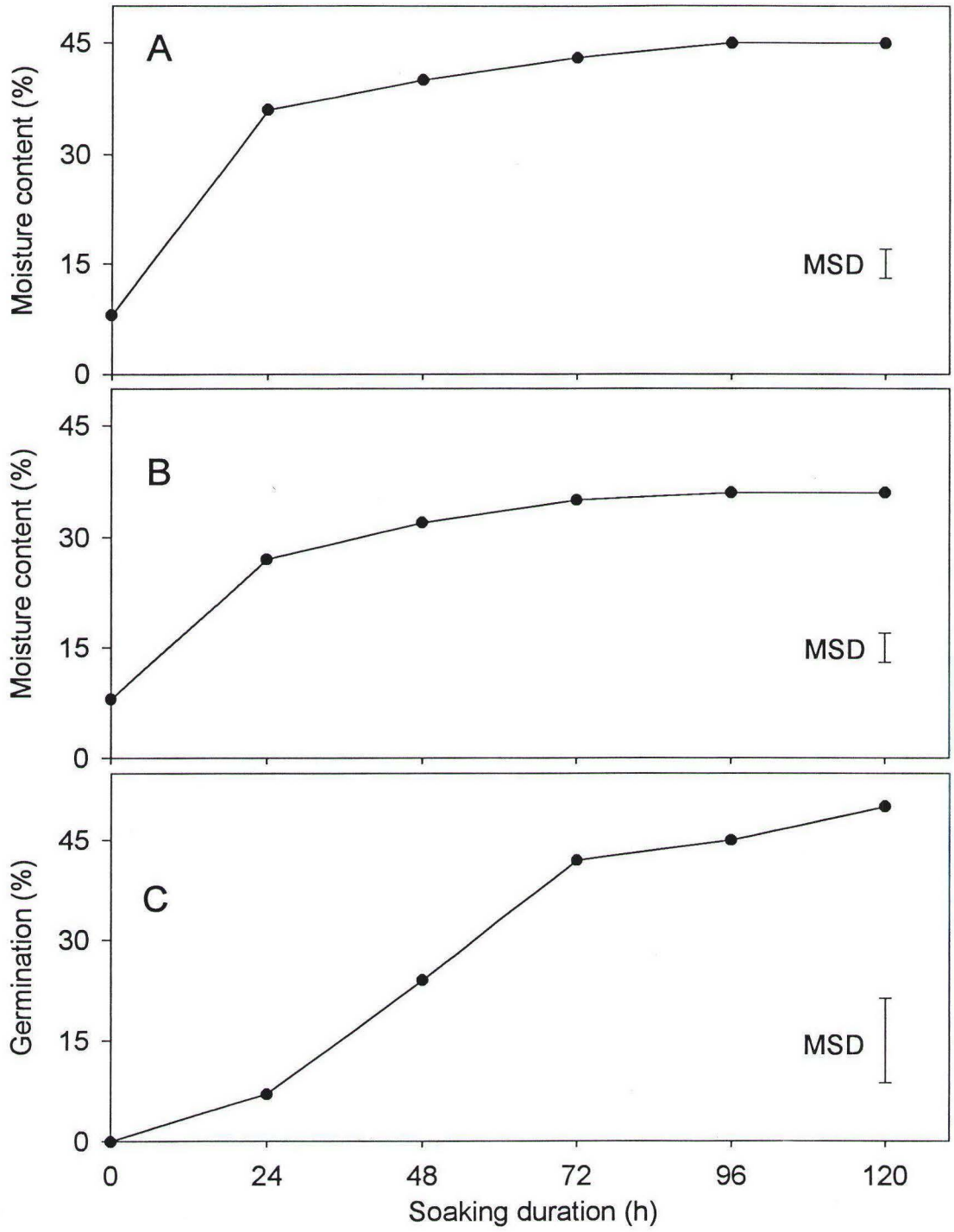

Figure 3.6. *Seed Moisture Contents and Resulting Germination.* Mean moisture contents of seeds after soaking (A) and after stratification (B), and resulting mean total germination (C) for MA 1993, 1994, and 1995 combined. Each moisture content mean is the average of three seedlots each replicated 10 times with 100 seeds per replicate. Each germination mean is the average of three seedlots each replicated four times with 50 seeds per replicate. Tukey's mean significant difference (MSD) was calculated at the 0.05 level of significance.

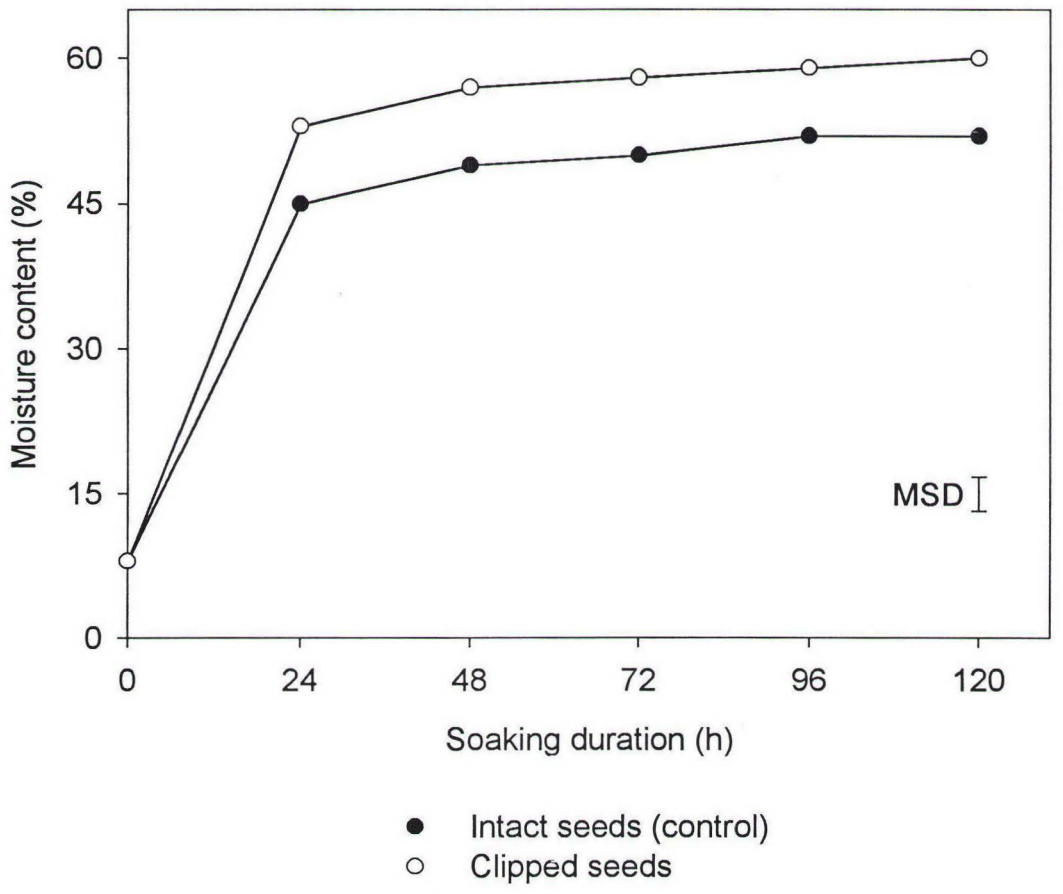

Figure 3.7. *Seed Moisture Contents of Intact and Clipped Seeds.* Mean moisture contents of intact and clipped seeds from MA 1993, 1994, and 1995 combined. Each mean is the average of three seedlots each replicated 10 times with 100 seeds per replicate. Tukey's mean significant difference (MSD) was calculated at the 0.05 level of significance.

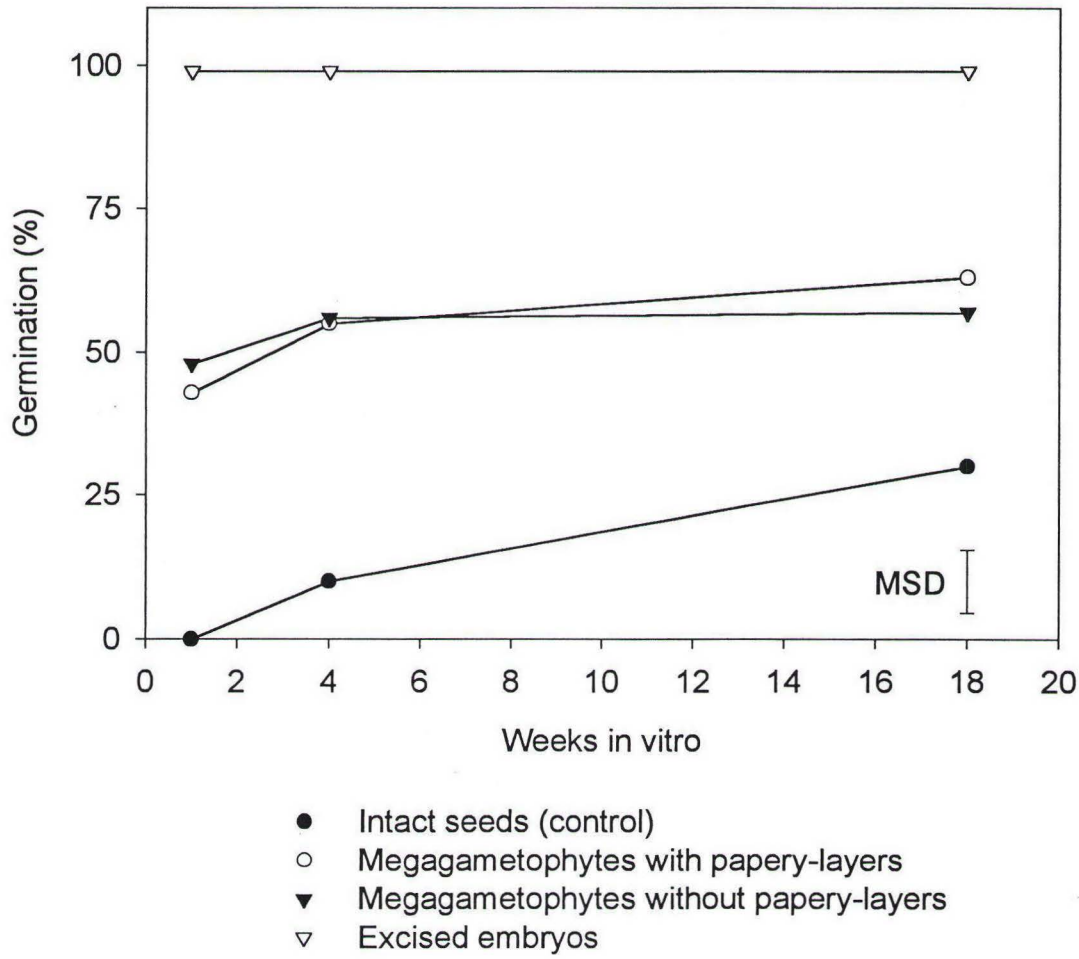

Figure 3.8. *Excised Embryos.* Average germination percentage of sterilized intact seeds, seeds with seed coats removed but most of the papery-layer intact, seeds with seed coats and papery-layers removed, and excised embryos after 1, 4 and 18 weeks on ½ Gresshoff and Doy medium. Seeds were nonstratified. Each mean is the average of three seedlots (MA 1993, 1994, and 1995) each replicated two times with 36 seeds per replicate. Tukey's mean significant difference (MSD) was calculated at the 0.05 level of significance.

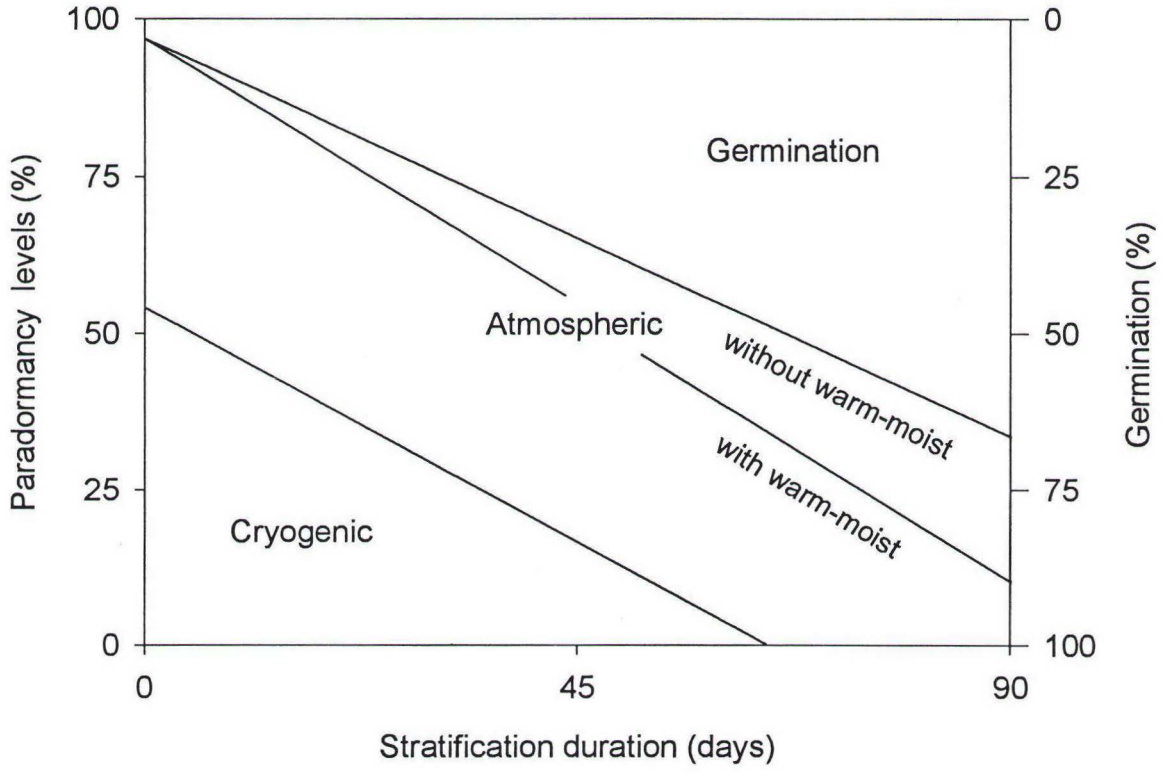

Figure 3.9. Schematic of paradormancy in *P. monticola* seeds.

APPENDIX

Tabular Data

Table 3.2. *Exposed Megagametophyte Placement.* For different stratification durations, mean total seed germination percentages of HC 15 and 31 after clipping and exposing megagametophytes to a moist substrate or toward air. Each mean is the average of three replicates of 20 seeds.

		Stratification duration (days)				
		0	21	42	90	120
HC Family	Placement	Germination (%)				
15	Toward air	85	96	96	100	100
	Toward moisture	74	65	74	92	100
	p value	0.4	0.006	0.003	0.13	-
31	Toward air	85	100	100	98	100
	Toward moisture	51	75	85	91	98
	p value	0.03	0.001	0.006	0.05	0.4

Table 3.3. *Warm-moist Treatment vs. Clipping.* Mean germination percentages of seedlots PCTC and 80-01 for different seed treatments by stratification duration. Each mean is the average of three replicates of 20 seeds.

		Stratification duration (days)		
		0	45	90
Seedlot	Seed treatment	Germination (%)		
PCTC	Control (stratified only)[1]	0 a[5]	15 a	32 a
	Clipped[2]	69c	86 c	82 b
	Warm-moist treated[3]	10 b	62 b	98 c
	Warm-moist & clipped[4]	75 c	82 c	94 c
80-01	Control (stratified only)	0 a	36 a	46 a
	Clipped	45 b	64 b	85 a
	Warm-moist treated	0 a	40 a	100 a
	Warm-moist & clipped	35 b	74 b	92 a

[1]Soaked 48 h in running 13°C water, then stratified at 3°C between moist Kimpaks.
[2]Soaked and stratified as above, then clipped as described by Hoff and Steinhoff (1986).
[3]Soaked as above, then incubated 10 days at 22°C, then stratified as above.
[4]Soaked, incubated, stratified, and then clipped as above.
[5]For each seedlot and stratification duration, means in columns with different letters are different at $p \leq 0.05$ using Tukey's studentized test.

Table 3.4. *Accelerated Aging.* Accelerated aging and clipping effects on mean total germination of seeds from 80-01 and MA 1990 and 1992 over different stratification durations. Each mean is the average of four replicates of 50 seeds.

Seedlot	Treatment	Stratification duration (days)			
		0	45	90	135
80-01	Control[1]	1 a[5]	5 a	31 a	30 a
	24 h[2]	3 a	10 a	34 a	15 b
	48 h[3]	5 a	34 b	48 a	34 b
	Clipped[4]	20 b	70 c	86 b	40 b
1990	Control	0 a	38 a	80 a	74 a
	24 h	0 a	49 ab	76 a	84 a
	48 h	2 a	66 bc	84 a	84 a
	Clipped	14 b	76 c	84 a	85 a
1992	Control	2 a	35 a	55 a	58 a
	24 h	1 a	48 ab	76 b	69 ab
	48 h	3 a	66 b	76 b	76 b
	Clipped	12 b	93 c	86 b	91 c

[1]Soaked 48 h in running 13°C water, then stratified at 3°C between moist Kimpaks.
[2]Soaked and stratified as above, then exposed to 40°C and 100% humidity in a Stults accelerated aging chamber for 24 h.
[3]Soaked and stratified as above, then 48 h accelerated aging as above.
[4]Soaked and stratified as above, then clipped as described by Hoff and Steinhoff (1986).
[5] For each seedlot and treatment, means in columns with different letters are different at $p \leq 0.05$ using Tukey's studentized test.

Table 3.5. *Seed Moisture Contents and Resulting Germination.* Mean seed weights of MA 1993, 1994, and 1995 before soaking, mean seed moisture contents after soaking in 13°C water for 24-120 h and after 90 days stratification, and resulting mean total seed germination. Each mean is the average of four replicates of 50 seeds.

Seedlot	Soaking duration (h)	Pre-soaking weight (g)	Seed moisture contents: Post-soaking (%)	Seed moisture contents: Post-stratification (%)	Germination (%)
1993	24	1.07[1]	38 a	28 a	4 a
	48	1.09	42 b	33 ab	18 b
	72	1.06	45 c	37 b	47 c
	96	1.10	46 c	38 b	49 c
	120	1.05	47 c	38 b	49 c
1994	24	1.00	36 a	28 a	14 a
	48	0.97	42 b	34 b	34 b
	72	0.97	45 bc	37 b	42 bc
	96	0.99	46 c	38 b	42 bc
	120	0.98	45 c	37 b	54 c
1995	24	1.06	34 a	25 a	4 a
	48	1.06	37 b	30 ab	20 b
	72	1.05	40 c	31 ab	36 bc
	96	1.06	42 d	33 b	44 c
	120	1.03	42 d	34 b	48 c

[1]For each seedlot and pre-soaking weight, moisture content or germination, means in columns with different letters are different at $p \leq 0.05$ using Tukey's studentized test.

Table 3.6. *Seed Moisture Contents of Intact and Clipped Seeds.* Mean seed moisture contents of intact and clipped seeds from MA 1993, 1994, and 1995 after soaking in 20°C water for 24-120 h. Each mean is the average of 10 replicates of 100 seeds.

Seedlot	Soaking duration (h)	Seed moisture contents: Intact seeds (%)	Seed moisture contents: Clipped seeds[1] (%)
1993	24	46 a[2]	56 a
	48	51 b	62 b
	72	52 bc	63 b
	96	54 c	64 c
	120	54 c	65 c
1994	24	47 a	53 a
	48	52 b	56 ab
	72	52 bc	58 b
	96	53 bc	58 b
	120	54 c	58 b
1995	24	42 a	49 a
	48	46 b	53 b
	72	47 bc	54 bc
	96	49 c	56 cd
	120	49 c	57 d

[1]The micropylar point of each seed was removed to expose megagametophyte as described in Hoff (1987).

[2]For each seedlot and seed treatment (intact or clipped), means in columns with different letters are different at $p \leq 0.05$ using Tukey's studentized test.